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P, 25,451

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PL 0173/0036 PA 278/8/1

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magnesium and aluminium salts. **Warnings and side effects** Gastro-intestinal disturbances are the side effects most commonly reported. A yellow to brown discolor
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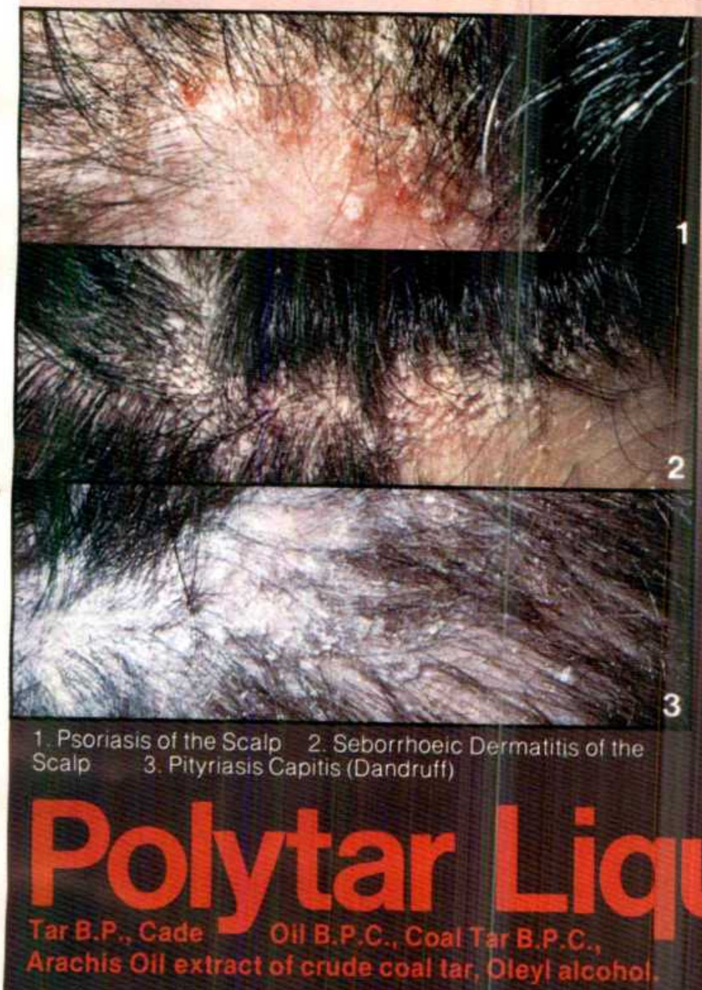
hydrochloride, supplied in bottles of 100 at a basic NHS cost of £20.23. PL 0095/0062. MINOCIN is a trademark. Full prescribing information is available on req

Lederle Laboratories Division of Cyanamid of Great Britain Ltd, Fareham Road, Gosport, Hants PO13 0AS (Tel: 0329-236131)

Common Disorders of the Scalp.



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Presentation	**Product Licence number**
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There will be 3 scientific sessions and a number of social engagements.

Any papers proposed to be presented should be sent to:

Dr M A Waugh
Hon Secretary MSSVD
Dept of Genito-Urinary Medicine
The General Infirmary
Leeds LS1 3EX

by Saturday, 22nd January 1983 at the latest, giving details of title, authors and a short summary of not more than 200 words, together with approximate duration of presentation. A Committee of the MSSVD will consider papers submitted and will let authors know in good time whether they are accepted.

The conference fee will be £60 for delegates and £30 for guests.

For further details please contact Dr M A Waugh

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The position will be available September 1982.

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Associate Professor/Full Professor

The Department is searching for an Associate Professor to Full Professor to serve as Director of the Dermatology Laboratories, and Co-Director of the NIH Training Grant. The position of Chief of the Veterans Administration Dermatology Service is optional.

The candidates must be board certified in Dermatology and have documentable expertise in Dermatologic research. Title, salary, equipment, and renovations are negotiable. Departmental funds are available to implement research activities. Research grants are available from the Veterans Administration Medical Center. The research area consists of 3,238 square feet of well-equipped laboratory space. There is also a possibility of a joint appointment with a basic science department.

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REFERENCE:
British Medical Journal, 1978, **2**, 84.

PL 0173/0030

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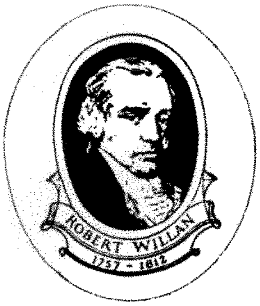

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Clinical and Laboratory Investigations

The analysis of the ultraviolet radiation doses required to produce erythemal responses in normal skin

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SUMMARY

When the abnormality of a skin response to sunlight is expressed in terms of a decreased minimum erythemal dose (MED) on photo-testing, it is essential to know the range of response in normal skin. In any homogeneous population, the statistical distribution of the MED is skewed and is not Gaussian (normal), but a Gaussian distribution can be fitted to the logarithm of the MED. Using established methods of probit analysis, such 'lognormal' distributions have been fitted to the observed proportions of erythemal responses to a series of test exposure doses of UV radiation (250–365 nm), thus providing estimates of the average log MED for normal skin, the standard deviations of the distributions and associated confidence limits. These estimates, in arithmetical terms, are available for quantitative comparison with the results of other test exposures on skin which may be abnormal.

In order to assess abnormal sensitivity of skin to UV radiation it is necessary to establish the response of normal skin to the same radiation, and to quantify the associated exposure-dose relationships. The factor to be considered in this paper is the production, or not, of a detectable erythemal reaction. The raw data for analysis were acquired in the Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, from monochromator irradiations of normal, lightly pigmented volunteers, selected to exclude those ingesting or applying medications. The xenon arc/monochromator units used have been described in a previous paper (Mackenzie & Frain-Bell, 1973). Having selected a central wavelength and bandwidth, the usual test procedure is first to irradiate the skin of the subject on several separate sites about 50 mm^2 in area, using a wide but coarse selection from a graded series of increasing exposure doses. This irradiation procedure is repeated at other central wavelength/bandwidth combinations. It is customary for a clinician to examine the irradiated sites visually, at about 7 and 24 h after the irradiation, when decisions are made as to whether or not an erythemal response is present at any part of the irradiated site, and, if so, to grade the response. A second

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course of irradiations is then given using finer but limited selections of exposure doses from the basic series, in order to obtain a more precise value of the minimum exposure dose which produces a discernible erythema at each particular waveband. This minimum dose is assigned as the minimum erythemal dose (MED). Clearly, the skin site with the next lower dose to the MED in the graded series does not show an erythemal response, and it follows that the true MED may in fact be lower than the exposure dose which is assigned to be the MED. In the Photobiology Unit, the same basic series of graded doses in steps of about 21% has been in use for over 10 years. This series of exposure doses (mJ cm^{-2}) uses the same numerical values as those for resistors in electronics, i.e. 1, 1·2, 1·5, 1·8, 2·2, 2·7, 3·3, 3·9, 4·7, 5·6, 6·8, 8·2, 10, 12, etc. Few authors specify their series of graded doses when publishing MED results, though this is of critical importance in determining the precision of an assigned MED. It could happen, for example, that for one test subject an erythemal response may be seen for the skin site given an incident exposure dose of 560 mJ cm^{-2} but not at 470 mJ cm^{-2}, the next lower dose. For a second test subject, irradiated only with a coarser selection of doses from the basic series, an erythemal response may also be seen at 560 mJ cm^{-2} but not at 270 mJ cm^{-2}, the next lower dose. The same MED, 560 mJ cm^{-2} would be assigned in both cases, but obviously the value for the first subject would be more precise. It should be noted that an assignment of an MED ignores responses to all test exposures except the two which straddle the true MED.

When combining the MED values from a group of normal subjects, it has been usual to calculate the arithmetic mean of the individual assigned MEDs without any account being taken of possible differences in precision. Two additional problems arise. First, how to include the results of those individuals whose graded series of test exposures did not extend far enough to straddle the MED. In these cases, the assigned MED would be given as 'greater than' or 'less than' the extremes of the graded dose series used. The second problem is that the estimated standard deviations associated with the calculations of arithmetic means often imply a range of likely normal MEDs extending down from the mean MED to zero exposure dose, e.g. mean 560 ± standard deviation 500 mJ cm^{-2}.

RE-ANALYSIS OF RESULTS OF TEST IRRADIATIONS

van der Leun (1972) stated that a sensitive and an insensitive Caucasian skin, both within the normal range, may well differ by a factor of ten in the dose of UV radiation required for reddening.

This suggested that the distribution of MEDs amongst normal individuals was unlikely to be Gaussian. It was decided to test the possibility that the distribution of the logarithms of the MEDs was Gaussian, i.e. a 'lognormal' distribution, by probit analyses of the logarithms of the available assigned MEDs from normal test subjects. The use of probits in effect converts the cumulative probability curve derived from a Gaussian distribution into a straight line, and is equivalent to plotting the observed cumulative proportions on probability graph paper. It must be emphasised that the use of the logarithm of the exposure dose and of the probit transformation are no more than convenient mathematical devices to allow the use of known methods of statistical analysis in the fitting of a regression line by the method of maximum likelihood. Any final statement of results must be in terms of the original exposure dose units and of proportions or percentages of subjects showing a response (Finney, 1964). Probit analysis partially solved the problem of dealing with MEDs quoted as 'greater than' some numerical value. Mather (1964) states that probit analysis is applicable to tests of the type in which the effect is 'quantal' and he details the method by means of an example involving the death or survival of experimental animals, sets of which are given a drug in a graded series of doses. Although the discernment of erythema of skin is not as definite or 'quantal' as death or survival, it was decided to re-apply probit analysis, not as previously to the assigned MEDs for each subject, but to *all* the test exposures made on *all* subjects. Thus for each test exposure dose, all results for subjects irradiated were combined to give an estimate of the probability of an erythemal response at that exposure dose.

In this way, not only was considerably more data included in the analysis, but the problem of variation in the precision of individual assigned MEDs was avoided, and the problem of MEDs 'greater than' or 'less than' some value was completely solved. The method described by Bliss (1935), Mather (1964) and Finney (1964), involving the use of 'working probits', enables the results from those exposure doses for which there were either no responses (o/n), or for which all subjects produced an erythemal response (n/n), to contribute also to the fitting of the regression line. If for a given test dose applied to n subjects, p subjects show an erythemal response at a particular exposure dose, then p/n is an estimate of the probability, P, of erythemal response. The variance of this estimate decreases as n increases, and also decreases the nearer p/n is to 50%. Each observed probit of p/n is therefore weighted in inverse proportion to its variance before the regression calculations are applied.

Probit analysis is well-known amongst biologists, and it is customary for them to make a logarithmic transformation of dose before applying the method. From the analysis, an estimate of the mean of the logarithm of the exposure dose can be calculated from which an arithmetical value can be obtained—compare the 50% effective dose (ED50) or 50% lethal dose (LD50) of the biologist. A statistical assessment can be made of how closely the logarithmic transformation of the exposure doses enables the observed data to fit a Gaussian distribution. For a near Gaussian distribution, the reciprocal of the slope of the probit regression line provides an estimate of the standard deviation of the population from which the sample was taken. This estimate, if sufficiently accurate, enables useful predictions to be made of the probability of an erythemal response at a particular exposure dose, or vice versa. In this paper, for example, the 95% confidence limits are given.

RESULTS

Data from normal subjects were available for xenon-lamp monochromator irradiations at the

TABLE 1. Wavebands used for tests of normal subjects

Central wavelength (nm)	Half maximum bandwidth (nm)	Filter
254	5	None
295	5	None
300	5	None
305	5	None
310	5	None
315	5	None
335	30	WG 305
365	30	WG 305

TABLE 2. Example of probit analysis data and conclusions (normal subjects, 305 ± 5 nm)

Exposure dose, D (mJ cm^{-2})	Log D	No. of erythemal responses (P)	Total no. of test exposures (n)	% (100 p/n)	Probit	Y visual	Working probit (Yw)	weight (nW)	Y calculated
15	1·18	0	15	0	—	1·62	1·35	0·07	1·53
18	1·26	0	1	0	—	2·08	1·77	0·02	1·99
22	1·34	0	20	0	—	2·53	2·17	1·07	2·46
27	1·43	0	17	0	—	3·03	2·60	2·35	2·98
33	1·52	2	49	4·1	3·26	3·54	3·31	13·83	3·50
39	1·59	5	37	13·5	3·90	3·93	3·90	15·35	3·90
47	1·67	18	45	40·0	4·75	4·38	4·78	24·87	4·36
56	1·75	13	42	31·0	4·50	4·83	4·52	26·44	4·82
68	1·83	37	57	64·9	5·38	5·28	5·28	35·25	5·29
82	1·91	30	39	76·9	5·74	5·73	5·74	20·39	5·75
100	2·00	7	9	77·8	5·77	6·24	5·62	3·21	6·27
120	2·08	4	4	100	—	6·69	7·17	0·84	6·73
150	2·18	2	2	100	—	7·25	7·64	0·17	7·31
	Totals	118	337						

Analysis of chi-squared

Item	Chi-squared	*n*	Probability
Regression	93·0	1	Very small
'Goodness of fit'	2·5	2	0·29

Log$_{10}$ average MED = 1·780 ± 0·015 (± 0·8%).
Regression line slope (b) = 5·78 ± 0·60 (± 10·4%).
Reciprocal of slope (1/b) = 0·173 ± 0·018.
= estimate of population standard deviation.
Log exposure dose 95% confidence limits = 1·44–2·12.
Conclusion: median MED = 60 mJ cm^{-2}.
95% Confidence limits = 28–132 mJ cm^{-2}.

wavelengths and bandwidths given in Table 1. All these wavebands were theoretically triangular in shape.

The full probit analysis data are given only for irradiations at one waveband 305 ± 5 nm (Table 2 and Fig. 1). As described by Mather (1964) and Finney (1964) the procedure is to plot the probit versus log exposure dose points and, from the straight line put through these points by eye, to calculate probit values, Y visual on this line corresponding with each exposure dose (Fig. 1). These values are then used to calculate, from tabulated data, the working probits, Yw, and weights nw, which form the basis of the regression analysis. A computer programme has been written incorporating the tables of relevant functions (Fisher & Yates, 1963) (reprinted in Documenta Geigy Scientific Tables, 1970). (The calculations can be carried out with a pocket

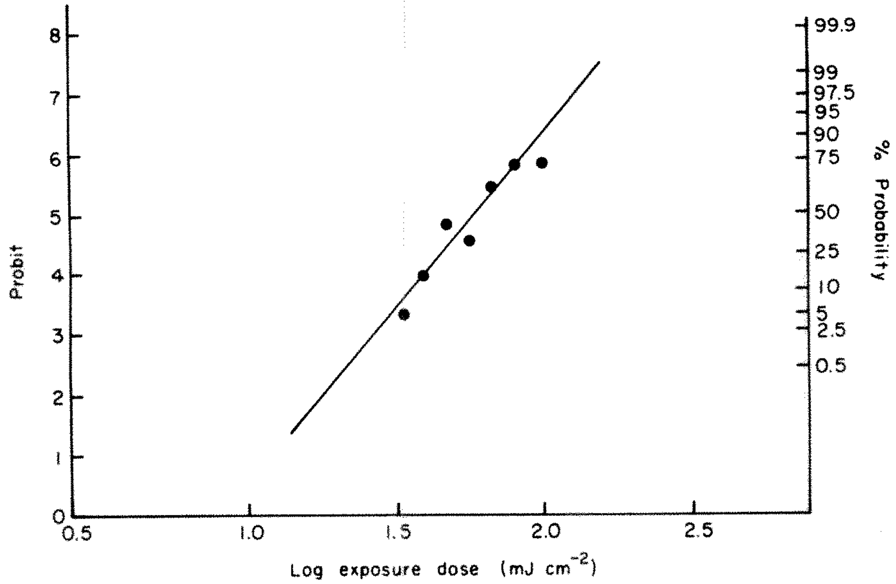

FIGURE 1. Example of probit versus log exposure dose regression line. Normal Subjects, 305 ± 5 nm.

calculator but they are lengthy and tedious and prone to mistakes.) It is advisable to use new probit values calculated from the first regression line to deduce a second set of working probits and weights and hence to reiterate the regression analysis. Usually the improvement of fit so produced is minimal but occasionally it can be significant. Reiteration for the data in Table 2 made only slight changes in the calculated parameters.

The analyses based on minimum discernible erythemas for the other wavebands in Table 1 were similar to that illustrated and the results are summarized in Table 3. In every case, the fitted regression line accounted for all but an insignificant portion of the variation in probit value.

TABLE 3. Summary of probit analysis results for minimal erythema in normal subjects

Central wavelength (nm)	Full bandwidth (nm)	Filter	Total no. test exposures	Log MED	Reciprocal of regression line slope (1/b)	Correlation coefficient (*r*)	MED (mJ cm^{-2})	95% Confidence limits (mJ cm^{-2})
254	± 5	—	80	0·64 ± 0·04	0·24 ± 0·04	0·98	4·3	1·5–13
295	± 5	—	131	1·10 ± 0·02	0·11 ± 0·02	0·98	12·5	8–21
300	± 5	—	102	1·43 ± 0·02	0·14 ± 0·02	0·97	27	14–51
305	± 5	—	337	1·78 ± 0·02	0·17 ± 0·02	0·95	60	28–132
310	± 5	—	113	2·44 ± 0·03	0·22 ± 0·04	0·86	270	113–644
315	± 5	—	51	3·06 ± 0·05	0·19 ± 0·06	0·96	1150	480–2,740
335	± 30	WG305	339	3·72 ± 0·02	0·26 ± 0·03	0·96	5250	1,600–17,300
365	± 30	WG305	192	4·17 ± 0·02	0·15 ± 0·02	0·98	14,800	7,600–29,000

DISCUSSION

Whenever there are more than three exposure doses, at each of which some, but not all, of about five or more subjects show an erythemal response, then probit analysis may be applied. Generally, of course, the analysis becomes more representative and more precise as the number of subjects and the number of exposure doses are increased. As has been shown, the probit method yields estimates of the logarithm of the average minimum erythemal dose, and of the standard deviation of the Gaussian distribution of log erythemal doses. Estimates of the standard errors of these two measurements can also be derived. From this information, fiducial limits, e.g. 95% confidence limits, can be estimated and expressed in terms of arithmetic values of exposure dose. Thereby, it is possible to assess the results of test exposures of patients whose skin may be abnormally photosensitive in terms of erythema production and to express quantitatively any deviation from the average normal response. The information also enables protocols and test exposure dose series for use in such diagnostic investigations to be more rationally based. The effects of potentially photosensitizing or photoprotective agents or conditions on normal skin can be investigated and quantified.

Although the number of test exposures for each waveband was in the range 50–340, the number of normal volunteers was, in retrospect, not as large or representative as desired. The results in Table 3 are therefore provisional. In particular, it remains to be confirmed whether or not the standard deviations of normal populations vary with central wavelength, although there is supporting evidence for this which is presented later.

Factors which might influence the distributions of erythemal doses from the irradiations described in this paper can be listed as follows:

(1) True variations, from a variety of causes, in the sensitivity of the skin amongst the subjects tested; the 'between-subject' variations.

(2) Variations due to the subjective assessments of the presence or absence of an erythemal response.

(3) Limitations set by the finite gradation of the basic series used in the selection of exposure doses.

(4) Variations in the ultraviolet dosimetry. Several thermopiles were used during the period and relatively small but significant inconsistencies between the thermopiles have been found, also long-term changes in the responsivity of individual thermopiles.

(5) Variations in the calibrations, settings and spectral purity of the several xenon arc/monochromators used for the test exposures over a period of several years.

Comparisons of the assessments of erythemal response by the several clinicians involved have not been possible.

The basic series of exposure doses increases in steps of about 21%, i.e. in log dose terms steps of about 0·08. This gradation is fine enough to allow estimates of the probit line slopes about five times those actually found and hence it is unlikely that the gradation of the basic series is a major factor in determining the estimated variations.

The estimates of the standard deviations of the distribution of log effective doses quoted in Table 3 range from 0·11 to 0·24, i.e. in arithmetic terms from ± 29% to ± 74%. Inconsistencies in UV dosimetry are very unlikely to have been as great as this, even over a period of several years.

The effects on the estimated standard deviations of long-term changes in the spectral output of the monochromators at particular settings of central wavelength and bandwidth have been

TABLE 4. Summary of probit analysis results for minimal erythema in selected 'normal' patients

Central wavelength (nm)	Full bandwidth (nm)	Period	No. of test exposures	Log MED	Reciprocal of regression line slope (1/b)	Correlation coefficient (r)	MED (mJ cm⁻²)	95% Confidence limits (mJ cm⁻²)
250	± 5	1,2,3	60	0·82 ± 0·07	0·34 ± 0·08	0·88	6·7	1·4–31
295	± 5	1	319	1·17 ± 0·02	0·25 ± 0·03	0·94		
		2	321	1·18 ± 0·02	0·23 ± 0·02	0·96		
		3	294	1·12 ± 0·02	0·22 ± 0·02	0·94		
		1,2,3	988	1·16 ± 0·01	0·24 ± 0·01	0·98	14	5–42
300	± 5	1	277	1·39 ± 0·02	0·20 ± 0·02	0·95		
		2	321	1·39 ± 0·02	0·21 ± 0·02	0·96		
		3	243	1·34 ± 0·02	0·28 ± 0·03	0·93		
		1,2,3	841	1·38 ± 0·01	0·23 ± 0·01	0·97	24	9–68
305	± 5	1	325	1·89 ± 0·03	0·27 ± 0·03	0·97		
		2	346	1·85 ± 0·02	0·23 ± 0·02	0·94		
		3	262	1·82 ± 0·03	0·23 ± 0·03	0·94		
		1,2,3	933	1·85 ± 0·01	0·24 ± 0·01	0·95	71	24–209
310	± 5	1	262	2·41 ± 0·03	0·26 ± 0·03	0·94		
		2	290	2·38 ± 0·04	0·32 ± 0·04	0·95		
		3	158	2·43 ± 0·07	0·40 ± 0·08	0·86		
		1,2,3	710	2·40 ± 0·02	0·30 ± 0·03	0·91	250	65 ± 975
315	± 5	1	286	2·95 ± 0·04	0·32 ± 0·04	0·93		
		2	308	2·93 ± 0·03	0·27 ± 0·03	0·95		
		3	207	2·98 ± 0·05	0·29 ± 0·05	0·87		
		1,2,3	801	2·95 ± 0·02	0·29 ± 0·02	0·98	890	240–3,300
320	± 5	1	144	3·40 ± 0·08	0·29 ± 0·07	0·90		
		2	199	3·42 ± 0·08	0·35 ± 0·07	0·89		
		3	131	3·31 ± 0·09	0·32 ± 0·08	0·66		
		1,2,3	474	3·40 ± 0·05	0·34 ± 0·04	0·95	2,510	540–11,700
325	± 5			Approx. 3·74			Approx. 5,600	
330	± 5			Approx. 3·86			Approx. 7,300	
305	± 15	1,2,3	384	1·50 ± 0·02	0·22 ± 0·02	0·97	32	12–85
335 (WG305)	± 30	1	330	3·74 ± 0·02	0·22 ± 0·02	0·96		
		2	294	3·73 ± 0·02	0·23 ± 0·02	0·96		
		3	224	3·78 ± 0·04	0·27 ± 0·04	0·87		
		1,2,3	848	3·74 ± 0·01	0·23 ± 0·01	0·98	5,500	1,950 ± 15,500
365 (WG305)	± 30	1	262	4·35 ± 0·04	0·25 ± 0·04	0·95		
		2	309	4·25 ± 0·03	0·26 ± 0·04	0·92		
		3	211	4·32 ± 0·05	0·28 ± 0·05	0·85		
		1,2,3	782	4·29 ± 0·02	0·25 ± 0·02	0·97	19,500	6,300–60,300

Period 1: July 1974–November 1976. Period 2: November 1976–January 1978. Period 3: January 1978–May 1981.

investigated by using the results from a group of about 150 patients who, although originally referred for skin photosensitivity tests, were subsequently classed as 'normal' in so far as erythemal response was concerned on the basis of clinical assessment as well as of the test results. This group includes about 7000 test exposures, covering the wavebands 250 ± 5, 295 ± 5, 300 ± 5, 305 ± 5, 310 ± 5, 315 ± 5, 320 ± 5, 325 ± 5, 330 ± 5, 305 ± 15, 335 ± 30 and 365 ± 30 nm, the latter three with WG305 filter. The wavebands were all triangular in shape except for the 305 ± 15 nm waveband which was nearly rectangular. Despite the obvious arguments against the use of the results from this pre-determined group, it was decided, because they cover a

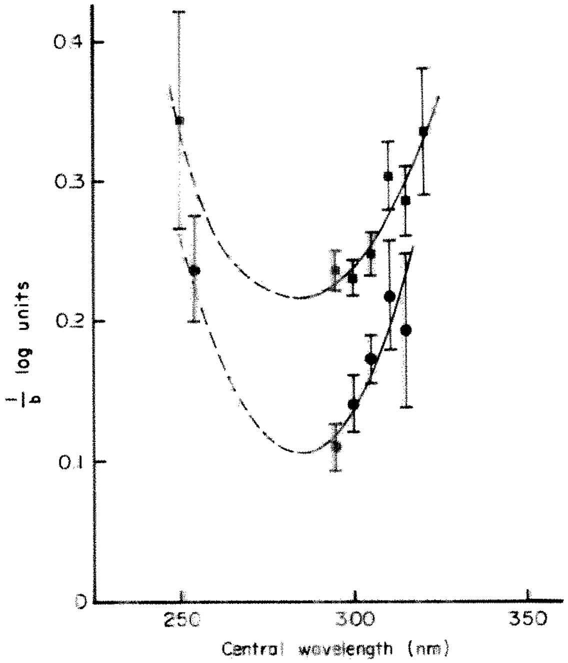

FIGURE 2. Variation of estimated population standard deviations with central wavelength. ■ = 'Normal' patients. ● = Normal subjects.

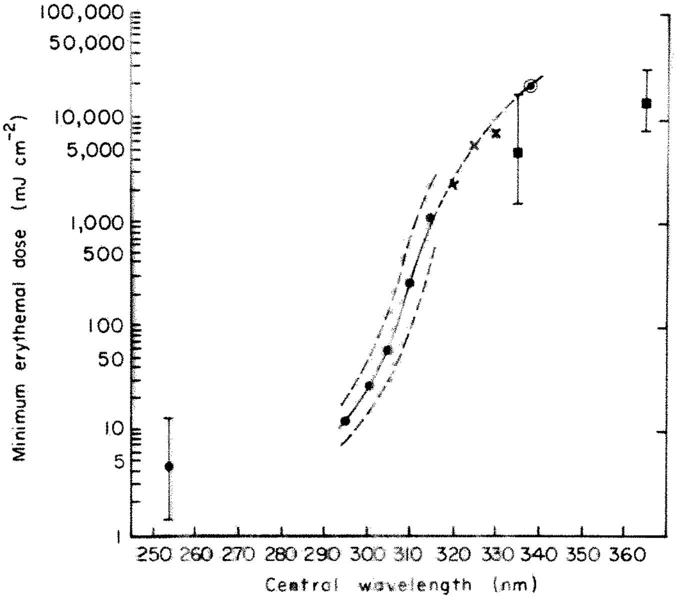

FIGURE 3. Minimum erythema doses with 95% confidence limits derived from probit analyses. ● = Normal subjects ± 5 nm. × = 'Normal' patients ± 5 nm. ■ = Normal subjects ± 30 nm. ⊙ The 337.1-nm laser line (Parrish *et al.*, 1976).

period of almost 7 years, to divide the group into three chronological subdivisions in an attempt to test for long-term consistency of the whole investigative system. The results are shown in Table 4. No significant trend in either log MED or 1/b with time is revealed.

Although the estimates of population standard deviations, 1/b, for the 'normal' patients are greater by about 30%, in arithmetic terms, than those for normal subjects, it can be seen that both groups show similar spectral curves for 1/b, with a minimum value at about 295 nm (Fig. 2). There thus appears to be less variation between the MEDs of individuals at 295 nm than at higher or lower wavelengths.

The MED results obtained by probit are summarized in Fig. 3, although it must be pointed out that the results given in this paper strictly refer only to the tests as carried out and assessed in the Photobiology Unit here. They may have no universal significance or application.

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I am indebted to Dr W.Frain-Bell, Consultant Dermatologist, for permission to use the data acquired in the Photobiology Unit, Department of Dermatology, Ninewells Hospital and Medical School, to Dr B.E.Johnson, Photobiologist, for discussions and encouragement, to Mr M.L.Wisbey, Senior Physicist, for the computer programme 'PROBIT', and to Professor Sir Kenneth Mather, for his generous help with the statistical analysis.

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The transmission of optical radiation through human nails

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SUMMARY

The transmission of optical radiation in the wavelength range 300–600 nm through thirteen human toenails obtained at autopsy was examined. The total transmitted radiation at fifteen different wavelengths was measured using an irradiation monochromator in conjunction with an integrating sphere and photodiode. A correction was included for fluorescence radiation from the nails.

The nails exhibited decreasing transmission of optical radiation in the wavelength region 600–300 nm. These findings may be helpful in the understanding of photo-onycholytic reactions and in the design of suitable regimens for the treatment of psoriatic nail changes.

The biochemical and biomechanical properties of nails are well established (Forslind, 1970; Baden, 1970), but there is little detailed information on the transmission of electromagnetic radiation through nails, particularly in the ultraviolet region (Gammeltoft & Wulf, 1980; Marx & Scher, 1980).

The mechanisms of photo-onycholytic reactions are unknown (Norton, 1980) but their eventual understanding will depend upon a knowledge of the optical radiation transmission characteristics of nails and a consideration of the action spectra and cellular mechanisms of the phototoxic compounds responsible. If photochemotherapy (PUVA) is to become a useful treatment for psoriatic nail changes, then effective regimens will have to take account of the transmission characteristics of nails.

The purpose of this work was to determine the transmission characteristics of optical radiation at wavelengths between 300 and 600 nm through human nails.

MATERIALS AND METHODS

Thirteen human toenails were obtained at autopsy. Grossly diseased or deformed nails were not

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selected as the object was to determine transmission through normal samples. The nails were removed whole, complete with the nail bed which was later separated by blunt dissection. Great toe nails were used as these provided a large area for the transmission studies. The mean thickness of the nails was 0·82 mm, with a range of 0·54–1·23 mm. The transmission of radiation through the nails at fifteen different wavelengths was carried out using an irradiation monochromator. The radiation was conducted from the exit slit of the monochromator by means of a liquid filled light guide. The distal end of the light guide was collimated to produce a circular field of radiation 3 mm in diameter. The total transmission of radiation through the nail was achieved by placing the nail over the entrance aperture of a integrating diffusing sphere coated internally with Eastman Kodak Diffuse Reflectance Standard Coating 6080 which results in a uniform spectral efficiency of response over the range of wavelengths 250–600 nm. A gallium arsenide phosphate (GaAsP) photodiode was mounted in a small aperture in the integrating sphere orthogonal to the entrance aperture. The radiation directly transmitted through the nail, i.e. the unscattered radiation, was measured by means of a collimated photodiode with an acceptance angle of ± 2° from the normal. The contribution of fluorescence radiation to the measured photocurrent was estimated by utilizing colour glass filters. The mathematical analysis developed to determine the fraction of the incident radiation at each wavelength transmitted through the nail is given in the appendix.

RESULTS

A theoretical expression for the fraction of radiation at wavelength λ nm transmitted through a nail of thickness x mm would be extremely complex and would require application of radiative transfer theory (Diffey, 1982). However, a simple expression based on Beer's law is adequate for the present purpose such that we can define an effective attenuation coefficient $\mu(\lambda)$ mm^{-1} which is related to the transmitted radiation by

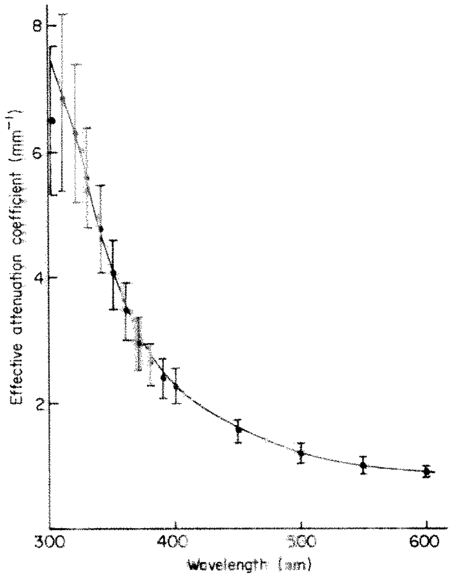

FIGURE 1. The effective attenuation coefficient for nails in the spectral range 300–600 nm.

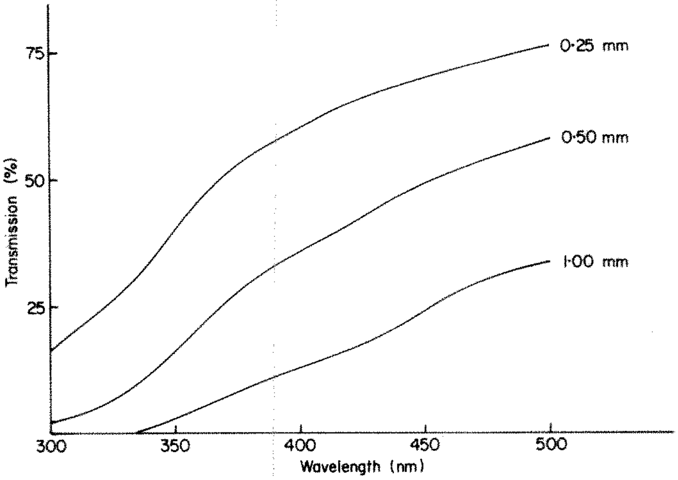

FIGURE 2. The transmission of optical radiation through nails of thickness 0·25, 0·5 and 1·0 mm.

$$\phi_x(\lambda)/\phi_0(\lambda) = \exp(-\mu(\lambda)x) \tag{i}$$

where $\phi_x(\lambda)$ is the photon flux at wavelength λ nm transmitted through a nail of thickness x mm and $\phi_0(\lambda)$ is the incident photon flux at wavelength λ nm. From the thirteen nails examined it has been possible to derive an effective attenuation coefficient as a function of wavelength and this is shown in Fig. 1. Attenuation of radiation is fairly flat in the visible region but rises sharply as the wavelength decreases in the ultraviolet region.

Figure 2 shows the total radiation transmitted through nails of thickness 0·25, 0·5 and 1·0 mm calculated by means of equation (i). Measurement of the fraction of incident radiation which was directly transmitted through the nails showed this to be considerably less than the total transmitted radiation, that is, the direct plus scattered radiation, implying that scattering is the dominant optical process in the nail.

DISCUSSION

Lewis and Montgomery (1955) suggested, on the basis of the distribution of 'senile' changes in elastic and connective tissue in the nail bed, that the nail offers only poor protection of the bed from exposure to sunlight. These observations have been disputed and our own findings suggest that the nail plate acts as a very efficient sunscreen, with very low transmission in the damaging UV-B range (280–315 nm).

Photo-onycholysis

Photo-onycholysis does not occur as an effect of sunlight alone, even in the presence of the most severe sunburn (Zala, Omar & Krebs, 1977), but it has been reported in association with a number of phototoxins, e.g. tetracycylines and furocoumarins (Zala *et al.*, 1977; Bethell, 1970; Rothstein, 1977; Rau, Flowers & Barett, 1978) and possibly chloramphenicol (Ray, 1963) and porphyrins (Byrne, Boss & Dawber, 1976).

The absorption spectrum of tetracycline is roughly constant throughout the entire UV spectrum and only begins to decrease appreciably at wavelengths greater than about 400 nm

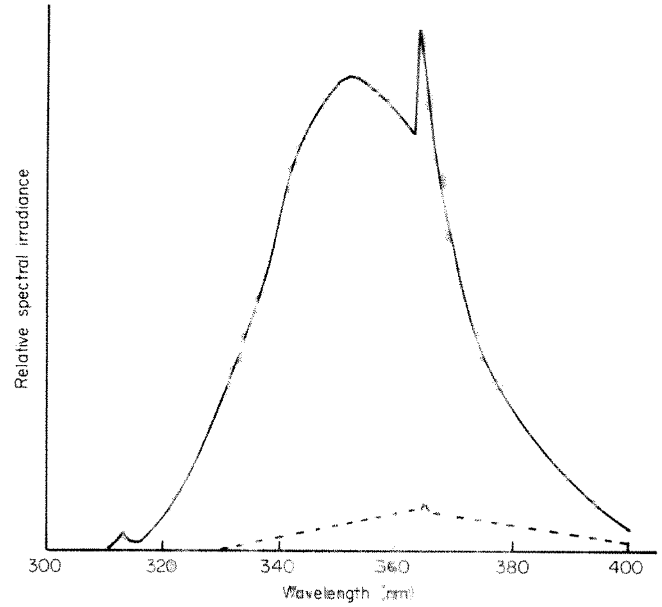

FIGURE 3. The spectral composition of radiation from a UV-A fluorescent lamp incident on (——) and transmitted through (- - -) a nail of average thickness (0·82 mm).

(Conover, 1956). The action spectrum for skin photosensitivity in patients with porphyria peaks at around 400 nm but can extend in some sensitive patients to 600 nm (Magnus, 1976). It is tempting to suggest, therefore, that only compounds with action spectra predominantly in the long-wave UVR and visible region would be capable of inducing phototoxic reactions beneath the nail plate. It is interesting to note that photo-onycholysis with the furocoumarins has only been reported following excessive exposure to UVR. The phototoxic action spectrum for this group of compounds peaks at around 330 nm but does extend up to about 380 nm (Nakayama *et al.*, 1974).

Photochemotherapy

Marx & Scher (1980) have suggested that oral psoralen photochemotherapy (PUVA) may be effective in the treatment of psoriatic nail changes, particularly those secondary to changes in the nail bed, hyponychium and the distal nail matrix, i.e. those regions which are accessible to irradiation. It is apparent from Fig. 2 that relatively little UV-A radiation reaches the nail bed. The spectral composition of the radiation which penetrates a nail of average thickness found in this study (0·82 mm) from irradiation with a standard UV-A fluorescent lamp is shown in Fig. 3. It is really only radiation with wavelengths greater than 330 nm which are transmitted, and radiation in the long wavelength end of the UV-A spectrum is possibly of limited efficacy in PUVA therapy compared with radiation at shorter wavelengths (Young & Magnus, 1981). The average transmission of UV-A radiation through the excised epidermis of Caucasian skin was found by Kaidbey *et al.* (1979) to be 55% (range 42–77%). This is appreciably greater than the 22% of incident UV-A which is calculated to reach a nail bed protected by a nail plate of 0·5 mm thickness. Therefore, assuming that the mechanisms for resolution of psoriatic nail changes are similar to those for psoriatic plaques, approximately 55/22, that is 2·5 times the therapeutic dose for glabrous skin would need to be delivered to the surface of the nail plate (with appropriate protection for the surrounding skin) to achieve the same effect. This factor assumes a flat action spectrum for the regression of psoriatic lesions due to psoralen photochemotherapy. If wavelengths around 330 nm are proved to be most effective therapeutically, the factor is likely to be closer to 5 because of the differing transmission characteristics of the epidermis and the nail plate. These figures have yet to be verified in clinical practice but they imply that specific photochemotherapy of psoriatic nails is a feasible, if potentially rather tedious, process.

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We are grateful to Professor S.Shuster for his helpful interest in this work.

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APPENDIX

Radiation of wavelength λ nm and photon flux $\phi_0(\lambda)$ incident at the entrance aperture of the integrating sphere will give rise to a photodiode current of $R_0(\lambda)$ mA, such that

$$R_0(\lambda) = \phi_0(\lambda)\rho(\lambda) \tag{Ai}$$

where $\rho(\lambda)$ mA is the response of the detection system per photon of wavelength λ nm.

When a nail of thickness x mm is placed over the entrance aperture, the observed photocurrent, $R(\lambda)$, will arise from two sources: radiation of wavelength λ nm which has been transmitted through the nail and fluorescence radiation in the blue region of the visible spectrum resulting from excitation of electrons in the nail (fluorescence radiation is only produced when the wavelength of the incident radiation is less than about 400 nm). Hence:

$$R(\lambda) = \phi_x(\lambda)\rho(\lambda) + F(\lambda) \tag{Aii}$$

where $\phi_x(\lambda)$ is the photon flux at wavelength λ nm transmitted through the nail of thickness x mm and $F(\lambda)$ is the contribution to the observed photocurrent from fluorescence radiation.

In order to estimate $F(\lambda)$ at each wavelength λ, a colour glass filter (Schott WG400) which transmits the fluorescence radiation but absorbs the incident ultraviolet radition was placed between the nail and the entrance aperture. The recorded photocurrent is now

$$R(\lambda)_{400} = gF(\lambda) \tag{Aiii}$$

where g is a factor which accounts for the geometry of measurement and reflection losses at the air/glass interfaces. This factor can be obtained by repeating the measurement using a filter (Schott WG280) which transmits both the fluorescence radiation and incident ultraviolet radiation. In this case the photocurrent is

$$R(\lambda)_{280} = g\phi_x(\lambda)\rho(\lambda) + gF(\lambda) \tag{Aiv}$$

so

$$g = R(\lambda)_{280}/R(\lambda) \tag{Av}$$

and

$$F(\lambda) = R(\lambda)\,.\,R(\lambda)_{400}/R(\lambda)_{280} \tag{Avi}$$

The required quantity, $\phi_x(\lambda)/\phi_0(\lambda)$, which is the fraction of incident photons of wavelength λ transmitted through the nail, is given by

$$\phi_x(\lambda)/\phi_0(\lambda) = \frac{R(\lambda)}{R_0(\lambda)}[1 - R(\lambda)_{400}/R(\lambda)_{280}] \tag{Avii}$$

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Ornithine decarboxylase induction in psoralen-treated mouse epidermis used as a test of UV-A sunscreen potency

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SUMMARY

Inhibition by sunscreens of UV-A-induced epidermal ornithine decarboxylase (ODC) activity in 8-methoxypsoralen-treated mice was used to examine the UV-A sunscreen activity of two commercial preparations, Uval® (para-aminobenzoic acid) and PreSun® (a benzophenone derivative). Both gave significant protection against UV-A doses of up to 3 J/cm^2 and Uval® was more effective that PreSun® in the UV-A range. At intermediate doses (5–15 J/cm^2) neither Uval® nor PreSun® significantly altered the ODC response to UV-A. At doses of 20–40 J/cm^2 UV-A, control and PreSun®-treated animals showed a decline in ODC activity which histological studies suggested was due to severe epidermal damage. This decline in ODC activity and severe epidermal damage was not seen in Uval®-treated animals. Topical zinc oxide was highly effective in preventing induction of ODC by UV-A irradiation at doses up to 40 J/cm^2, and was significantly more effective than either PreSun® or Uval®.

Sunscreens are commonly used to try to prevent the immediate and long-term damaging effects of sunlight upon normal skin. The prevention of painful erythema is the most striking benefit. Erythema is the most widely used means of assessing sunscreen potency for this reason, and also because it is a reproducible cutaneous response which may be assessed non-invasively. Most of the erythemogenic radiation from the sources commonly used for testing lies in the UV-B region (Parrish *et al.*, 1978; Sayre *et al.*, 1979). This is true both for natural sunlight and for the output of a solar simulator. It follows that sunscreen protection against UV-B rather than against UV-A is measured by this type of assay.

It is becoming increasingly recognized that longwave UV radiation (UV-A) may have effects upon normal skin, including immediate (Pathak *et al.*, 1962) and delayed (Pathak & Epstein,

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1971) tanning and histological damage (Willis & Cylus, 1977) as well as erythema, although UV-A is much less erythemogenic than UV-B. The use of UV-B sunscreens may allow greatly increased UV-A exposure of normal individuals. UV-A is also important in a variety of disease states including chemical phototoxicity and photoallergy, persistent light reactions, actinic reticuloid, some cases of polymorphous light eruption, solar urticaria, endogenous photosensitization syndromes, melasma and ephelides. A role therefore exists for sunscreens effective against UV-A, but measurement of biological effectiveness is difficult. UV-A erythema requires high intensity sources and exposure times may be long. The ability of sunscreens to prevent the erythema response to photosensitizers whose action spectrum lies within the UV-A region has been assessed (Parrish, Pathak & Fitzpatrick, 1971).

Our observation that UV-A induces high levels of epidermal ornithine decarboxylase (ODC) in mice topically photosensitized with 8-methoxy-psoralen (Gange & DeQuoy, 1980; Gange, 1981) has provided an alternative marker by which sunscreen potency against UV-A may be evaluated. The effect of two sunscreen preparations upon this response is presented.

MATERIALS AND METHODS

Female hairless albino HRS/J mice between 2 and 4 months of age were used for all experiments.

Light source

Four F40 BLB lifeline fluorescent tubes (Sylvania) were mounted 12·5 cm apart in a foil-lined holder; animals were irradiated at a distance of 55 cm in a specially designed compartmentalized cage. This provided a dose of 0·12 J/cm²/min, measured using an LM301 UV-A meter (National Biological Corporation), with peak emission at 366 nM.

8-Methoxypsoralen (8-MOP) treatment

Approximately 8 sq cm of the dorsal skin of each animal was painted with 0·02 cc of a preparation of 1% 8-MOP in an alcohol/propylene glycol base (Oxsoralen®, Elder). Animals were then immobilized for 60 min before sunscreen application.

Sunscreen preparations

Two proprietary sunscreen preparations were evaluated: PreSun® lotion (Westwood Pharmaceuticals), containing 5% p-aminobenzoic acid, absorbs predominantly in the UV-B region; Uval® sunscreen lotion (Dorsey Laboratories) contains 10% 2-hydroxy 4-methoxy-benzophenone-5-sulphonic acid and it absorbs both in the UV-B and UV-A regions. Zinc oxide ointment USP was also tested as an example of a physical blocker.

The sunscreen preparation (0·04 cc) was carefully and evenly spread over the psoralen-treated skin and the animal was immobilized for 15 min before irradiation. Zinc oxide ointment was applied immediately before irradiation; an amount producing a visible coating (0·04 cc) was measured from a tuberculin syringe.

Ornithine decarboxylase assay

Enzyme activity was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine by the method of Russell & Snyder (1968) with minor modifications.

Enzyme preparation. Animals were killed 24 h after irradiation by neck dislocation. The dorsal skin was removed immediately. Epidermis was separated by immersion of the skin in 50 mM phosphate buffer at 56°C for 15 s, followed by immediate cooling in buffer at 0°C; the skin was then spread on paper, epidermis upward. The epidermis was blotted dry, removed by gentle scraping and placed in preweighed tubes surrounded by ice. In each experiment skins were assayed either singly or in pairs; results are the mean of four or five animals per experimental group. After the tubes were weighed, the epidermis was homogenized for 15 s in 19 vol. of 50 mM phosphate buffer containing 0·2 mM pyridoxal phosphate, 1 mM EDTA, and 4 mM dithiothreitol, using a Brinkmann homogenizer. The homogenate was centrifuged at 20,000 ***g*** for 10 min at 0°C, giving a clear supernatant.

Ornithine substrate. Ornithine hydrochloride (1·2 mM) was dissolved in buffer containing pyridoxal phosphate, EDTA and dithiothreitol as above. L-[1-^{14}C]ornithine hydrochloride 0·25 μCi/0·1 ml substrate was added.

Procedure. Enzyme preparation (0·2 ml) was put in each 15 ml tube. Labelled substrate (0·1 ml) was added and the tube closed with a rubber stopper fitted with a central well assembly (Kontes) containing a filter paper bearing 0·05 ml NCS tissue solubilizer (Amersham), for CO_2 absorption. The tubes were incubated for 60 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 0·2 ml 2M citric acid. Incubation was continued for 1 h to ensure complete absorption of $^{14}CO_2$ by the NCS. Filter papers were then transferred to scintillation vials containing 5 ml of Beta-Max (Amersham). Blank tubes contained no enzyme. Release of $^{14}CO_2$ was linear up to 1 h. Results are expressed as nM CO_2/10 mg tissue/h. Data presented represent the pooled results of between two and nine experiments per point.

Histopathological studies

In some experiments, 6 mm punch biopsies were removed from the centre of the dorsal skin sample before heat treatment. The specimens were processed and stained with hematoxylin and eosin for light microscopy. The degree of epidermal damage was graded 'blind' by two independent observers: 0, normal epidermis; 1, minimal vacuolization and/or scattered necrotic epidermal cells; 2, patchy necrosis of epidermis with infiltration of inflammatory cells; and 3, complete epidermal necrosis. For each specimen the grades estimated by each observer were averaged, the results were expressed as the mean for each group of five animals.

Experimental design

Groups of five animals treated with 8-MOP alone were killed at intervals up to 48 h after UV-A irradiation, to determine the time at which ODC activity was maximal.

The effect of UV-A dosage upon ODC induction at 24 h was determined by irradiating groups of 8-MOP-treated animals with UV-A dosages ranging from 0·75 to 40 J/cm^2. Animals treated with sunscreen as described were irradiated with the same UV-A dosages concurrently and ODC induction was compared with animals receiving no sunscreen treatment.

To control for effects of sunscreens upon ODC induction which could be unrelated to UV-blocking activity, groups of animals treated with 8-MOP and UV-A irradiation were treated with sunscreens immediately after irradiation: these were compared with animals receiving no sunscreen application.

RESULTS

Animals treated with 8-MOP and UV-A first showed increased epidermal ODC activity at 14 h. This reached a maximum at 24 h and then levels slowly declined but were still elevated at 72 h.

In all experiments employing sunscreens, animals were killed at 24 h. 8-MOP alone, or UV-A alone at doses up to 40 J/cm^2 caused no elevation of ODC activity, confirming that the small UV-B emission from this source was not sufficient to interfere with the experiment.

ODC induction was not observed at UV-A doses of 0·75 J/cm^2 or less; between 1·5 J/cm^2 and 5 J/cm^2 ODC induction increased with UV-A dosage (Fig. 1); a plateau was observed between 5 and 15 J/cm^2; above 15 J/cm^2 ODC activity decreased with increasing UV-A dosage.

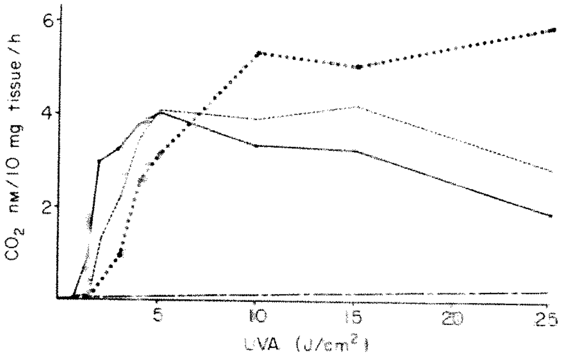
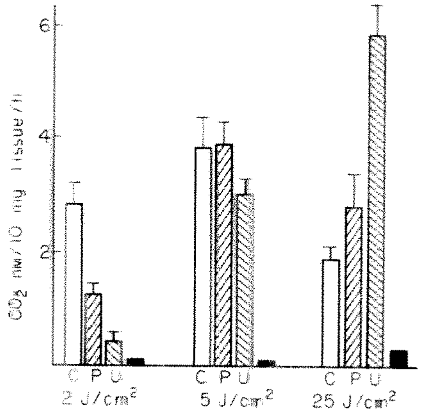


FIGURE 1. ODC activity in control and sunscreen treated animals plotted against UVA dose. Uval (· · ·); PreSun® (---); control (——); zinc oxide (– – –).

Sunscreen application increased the minimum doses of UV-A necessary to cause induction of ODC. Animals without sunscreen showed ODC induction after 1·5 J/cm^2 UV-A, while no significant activity was seen in animals treated with either sunscreen at this dosage.

At 2 J/cm^2 UV-A (Fig. 2), both sunscreens significantly lowered ODC induction (PreSun® vs. control $p < 0·005$, Uval® vs. control $P < 0·001$). Uval® was significantly more effective than



	P	*n*	*P*	*n*	*P*	*n*
C/P	<0·005	13	n.s.	31	n.s.	14
C/U	<0·001	13	n.s.	25	<0·001	17
U/P	<0·001	10	n.s.	26	<0·005	15

FIGURE 2. ODC activity at representative points in the low, mid, and high UV-A dose ranges, in control, sunscreen and zinc oxide treated animals. *P* values are given for control vs. PreSun® (C/P), control vs. Uval® (C/U) and Uval® vs. PreSun (U/P). Uval (□); PreSun® (▨); control (▨); zinc oxide (■).

PreSun® ($P < 0·001$). ODC levels were significantly lower than in PreSun®-treated or control groups at UV-A doses up to 4 J/cm^2.

A sharp increase in ODC induction occurred in all groups with increasing UV-A dosage. Sunscreen-treated and control animals did not differ significantly at UV-A doses of 5–15 J/cm^2 (Fig. 2).

At higher UV-A doses (20–40 J/cm^2), Uval®-treated animals continued to show high levels of ODC activity. Values in PreSun®-treated and control animals showed a decline in activity and did not differ significantly from each other, but were significantly lower than Uval®-treated animals (Fig. 2).

Zinc oxide effectively blocked ODC induction at all UV-A doses tested.

PreSun®, Uval® and zinc oxide had no effect upon ODC activity if applied to previously irradiated animals: this is consistent with their effect being related to UV-A blocking ability rather than mediated by some other mechanism.

Histological studies

Animals treated with 8-MOP + 15 J/cm^2 UV-A with or without sunscreen application were compared with respect to the degree of epidermal damage present, to see if this could be correlated with the fall off in ODC activity seen in PreSun®-treated animals and animals receiving no sunscreen application at this UV-A dosage. Animals receiving UV-A only were also assessed.

Gradings of epidermal damage estimated by two observers correlated closely: the results are summarized in Table I.

Uval®-treated animals showed significantly less epidermal damage than either PreSun®-treated animals or control animals receiving 8-MOP + UV-A only. PreSun®-treated animals did not differ significantly from controls. Animals receiving UV-A only (15 J/cm^2) were each graded 0 by both observers.

TABLE I. Epidermal damage in sunscreen-treated, positive control, and negative control groups (for grading see text)

	Positive control (8-MOP + UV-A)	8-MOP + UV-A + PreSun®	8-MOP + UV-A + Uval®	Negative control (UV-A only)
Degree of epidermal damage	$2·6 \pm 0·18$	$2·4 \pm 0·23$	$1·0 \pm 0·22$	0 ± 0
	└── n.s.	──┘		
		└ $P < 0·01$	┘	
	└── $P < 0·005$	────	──┘	

DISCUSSION

Clinical impressions of the value of non-opaque sunscreens as UV-A blockers suggest that they are relatively ineffective when compared with their high efficiency against UV-B.

Although the ability of sunscreens to absorb longwave UV *in vitro* is easily measured, assay of biological effectiveness is more difficult and in the past has relied on the measurement of

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UV-A-induced delayed erythema (Parrish *et al.*, 1978), or erythema induced by UV-A in psoralen photosensitized individuals (Parrish *et al.*, 1971). The induction of ODC by UV-A and 8-MOP (Gange & DeQuoy, 1980) may be an alternative marker by which protective agents can be measured. ODC is the rate-limiting enzyme in polyamine biosynthesis (Russell & Snyder, 1968), and elevated levels of the enzyme are usually associated with tissue proliferation (Janne & Raina, 1968; DiPasquale, White & McGuire, 1978; Mamont, Bohlen, McCann, Bey, Schubek & Tardif, 1976; Morrison & Goldsmith, 1978). Increased ODC activity in epidermis may be induced by a variety of stimuli including hair plucking (Probst & Krebs, 1975), the application of tumour-promoting agents (O'Brien, Simsiman & Boutwell, 1975), wounding (Clark-Lewis & Murray, 1978) UV-B and UV-C irradiation (Lowe, Verma & Boutwell, 1978). All these stimuli cause increased epidermal or follicular DNA synthesis. In UV-B or UV-C irradiated skin, this results in epidermal thickening and scaling.

The significance of ODC induction in phototoxic reactions is not clear, but could be related to cell proliferation in the repair process which follows epidermal damage. ODC induction in 8-MOP photosensitized animals differs from that occurring after UV-B or UV-C irradiation in being delayed for 14 h after irradiation.

Our data show that in the 8-MOP photosensitized hairless mouse treated with small amounts (2 J/cm^2) of UV-A irradiation, treatment with either of two proprietary sunscreen preparations before irradiation is effective in reducing at least the ODC induction component of this phototoxicity reaction. The benzophenone preparation (Uval®) was significantly more effective than PABA (PreSun®).

The effect of the sunscreens at blocking ODC was not apparent at UV-A doses between 5 and 15 J/cm^2, suggesting that the UV-A blocking effect of the sunscreen is easily overcome. These UV doses are comparable with those occurring in the course of routine sun exposure. The great majority of solar UV radiation energy lies in the longwave (> 320 nM) region and may amount to 9 J/cm^2/h when the solar zenith angle is 45° (Parrish *et al.*, 1978). Zinc oxide on the other hand was highly effective at blocking UV-A and virtually no induction of ODC occurred in the animals treated with this material.

At higher doses of UV-A (20–40 J/cm^2), control animals treated with 8-MOP but no sunscreen showed less ODC activity than animals receiving intermediate doses. PreSun®-treated animals showed a similar decline in ODC activity with higher UV-A doses, and did not differ from controls. We have observed a similar diminution in ODC activity when mice are treated with large doses of UV-B in the absence of a photosensitizer: under both circumstances the skin appears visibly damaged. In contrast, animals treated with Uval® do not show a decline in ODC activity at high UV-A doses.

The histopathological studies demonstrate extensive epidermal damage at this dose range, both in control and PreSun®-treated animals. Much less damage was observed in the animals pretreated with Uval®. This reduction of morphological changes in the epidermis, and continued high level of ODC activity, suggest that this sunscreen may protect against the severe epidermal damage resulting from 8-MOP phototoxicity. The lack of effect of Uval® upon ODC induction if applied immediately after high dose UV-A irradiation suggests that this effect is related to UV-A blocking activity rather than to an anti-inflammatory or other type of effect.

We have shown that ODC induction can be used to demonstrate the ability of a sunscreen to block low doses of UV-A and can discriminate between the effectiveness of two different sunscreen preparations. Our results agree with clinical impressions that non-opaque sunscreens are relatively ineffective against physiological doses of UV-A when compared with an opaque agent such as zinc oxide.

Since the action spectrum for 8-MOP photosensitized ODC induction is not known, the spectral sensitivity of this assay cannot be determined. If the action spectrum for ODC irradiation parallels that of other aspects of cutaneous psoralen phototoxicity such as erythema or sunburn cell formation, the assay is likely to be most sensitive to sunscreening effectiveness in the shorter part of the UV-A region, although the broad peak of emission of the fluorescent tubes at 366 nm will also exert an influence.

ACKNOWLEDGMENT

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Improvement of the hairless mouse screening model for antipsoriatic drugs

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SUMMARY

The hairless mouse screening model for antipsoriatic drugs was improved by investigating the effects of critical variables on the rate of ultraviolet light-induced epidermal DNA synthesis. The variables investigated were diurnal variation, ultraviolet light dose, time course and ambient temperature. From these studies a set of experimental conditions was developed that resulted in the lowest rate of DNA synthesis in non-irradiated epidermis and the highest rate in irradiated epidermis, thereby significantly increasing the power of the assay.

Irradiation of skin with ultraviolet light results in epidermal hyperplasia (Soffen & Blum, 1961; Epstein, Fukuyama & Fye, 1970). This observation has led to the development of the hairless mouse screening model for antipsoriasis drugs (du Vivier & Stoughton, 1975; du Vivier *et al.*, 1976). This model is useful for evaluating topical and systemic drugs for their antiproliferative properties in the epidermis. The applicability of this model to psoriasis is suggested by the observations that drugs known to be effective in psoriasis inhibit the ultraviolet light-induced increase in (1) incorporation of [^{3}H]-thymidine into DNA, (2) mitotic index, and (3) labelling index.

During our experiences with this model over the last few years, we have found that small variations in experimental procedures can lead to large differences in the quality of the results. Therefore, we have carefully and systematically reinvestigated the independent variables in this model with the goal of optimizing the experimental protocol. We report here the results of these studies.

MATERIALS AND METHODS

Hairless mice, strain Skh:HR (type 1), from Temple University, were used in all experiments.

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Ultraviolet irradiation was provided by a bank of two fluorescent sun lamps (Westinghouse FS-20 or FS-40) at a distance such that the UV-B energy at the peak of the mouse's back was about 10^{-4} W/cm^2. The mice were restrained in a wire cage under the lamps for different periods of time. As an alternative to the cages, the mice were anaesthetized with 0·1 ml of a 100 mg/ml sterile chloral hydrate solution and laid under the lamps for different periods of time. At varying times after the ultraviolet light exposure, 25 μCi of [^{3}H]-thymidine (0·1 ml, 6·7 Ci/mmole, New England Nuclear) was injected intraperitoneally. One hour later the animals were killed by cervical dislocation. The skin of the back was quickly cut off, immersed in a water bath at 55–65°C for 30 s, blotted dry and scraped lightly with a razor blade to remove the epidermis. The epidermis was then wrapped in aluminium foil, frozen on dry ice and stored at −20°C. When required, a $\frac{1}{4}$-inch arch punch was used before heat treatment to obtain a punch biopsy, which was stored in a 10% buffered formalin solution until processing. Histological samples were fixed in paraffin blocks and stained with hematoxylin and eosin.

To extract DNA from epidermis, the following buffers were used: buffer A, 8 M urea, 0·24 M sodium phosphate, 1% SDS and 1 mM EDTA; buffer B, 8 M urea and 0·24 M sodium phosphate; buffer C, 0·14 M sodium phosphate; buffer D, 0·48 M sodium phosphate. The sodium phosphate component of each buffer is equimolar parts of the monobasic monohydrate and the anhydrous dibasic salts. The tissue (40–70 mg) was weighed and homogenized in 5 ml of buffer A with a motor-driven, Teflon-glass tissue grinder. The homogenate was loaded onto a hydroxyapatite column (HPT, BioRad) constructed in a 20-ml syringe. The column consisted of a cloth disc and a 3–5 mm layer of sand supporting 3–5 ml of swollen hydroxyapatite. The column was washed successively with 90 ml each of buffers B and C. The DNA was then eluted from the column with 6 ml of buffer D. The first millilitre of eluate was discarded and the next 4 ml, containing the DNA, was collected. A 1-ml aliquot of the DNA solution was mixed with 5 ml of Scintisol (Isolab, Inc.) and counted in a liquid scintillation spectrometer. Absorbance at 280 and 260 nm was determined using the remainder of the DNA solution. The 260/280 ratio must be at least 1·8 or the DNA is judged to have significant protein contamination. The amount of DNA was quantitated by assuming that 1 absorbance unit at 260 nm represents a DNA concentration of 50 μg per ml. The apparent rate of DNA synthesis was measured by calculating the c.p.m. of [^{3}H]-thymidine incorporated per 10 μg of DNA.

Diurnal variation in the rate of epidermal DNA synthesis

The rate of epidermal DNA synthesis at different times of the day was measured. The object of these experiments was to find the time of day when the normal rate of DNA synthesis was the lowest. If the rate of ultraviolet light-induced DNA synthesis could be measured at the time when the normal rate of DNA synthesis was the lowest, then the largest difference between the rates of DNA synthesis in the irradiated and non-irradiated controls could be obtained. Increasing the difference between these two rates would be expected to increase the capability of the assay for detecting small degrees of inhibition. The results of these studies are summarized in Fig. 1. Clearly, the rate of DNA synthesis is the lowest at 13:30 (240 c.p.m./10 μg DNA) and is only about one-half that measured at 07:30 h (490 c.p.m./10 μg DNA). Under the conditions of these assays, the rate of DNA synthesis 48 h after irradiation was about 1200 c.p.m./10 μg DNA when measured at either 07:30 or 13:30 h. Therefore, the differences between the irradiated and non-irradiated controls were about 35% greater when the measurements were taken at 13:30 h than when they were taken at 07:30 h. On the basis of these results, all subsequent experiments were designed so that the rate of DNA synthesis was measured at about 13:30 h.

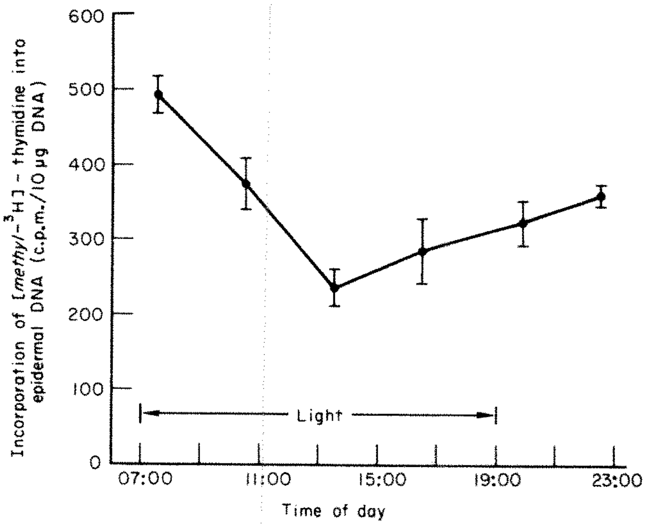

FIGURE 1. Diurnal variation in the rate of epidermal DNA synthesis. The animals were kept at a temperature of 24 ± 0·5°C with light between 07:00 and 19:00 h. At various times of day, groups of five animals were killed, and the incorporation of [methyl-^{3}H]-thymidine into epidermal DNA was determined. The values reported are the mean values for the group of five animals; the bars represent the standard errors.

Effect of the dose of ultraviolet light on the rate of epidermal DNA synthesis

The goal of these experiments was to find a dose of ultraviolet light that resulted in the maximal increase in the rate of DNA synthesis over non-irradiated controls. Groups of five mice were each held in wire-restraining cages and irradiated for different lengths of time. The rate of DNA synthesis was measured 48 h later. The results are represented by the dashed lines in Fig. 2. Under these conditions, 10–12 min of ultraviolet irradiation produced the maximum increase in the rate of epidermal DNA synthesis. Longer periods of irradiation resulted in less DNA synthesis.

Even though the animals were held in restraining cages during irradiation, they succeeded in moving around and often turned upside down. Therefore, we became concerned that this movement would result in variation of the total light dose from animal to animal. To prevent this movement, we anaesthetized the mice with chloral hydrate and irradiated them for different lengths of time. The results are represented by the solid lines in Fig. 2. Under these conditions, approximately 7 min of ultraviolet irradiation produced the maximum increase in the rate of DNA synthesis. As in the above experiment, longer periods of irradiation resulted in a decreased rate of DNA synthesis.

Histological examination of the epidermis from animals irradiated for 10 min in restraining cages while conscious and from animals irradiated under anaesthesia for 7 min, showed that in both cases the epidermis was two to three times thicker than that from non-irradiated animals. In addition, the mitotic index was approximately threefold greater in the irradiated animals than in non-irradiated controls.

On the basis of these results all subsequent experiments were performed using animals that were irradiated for 7 min under anaesthesia at a light dose of 10^{-4} W/cm^2.

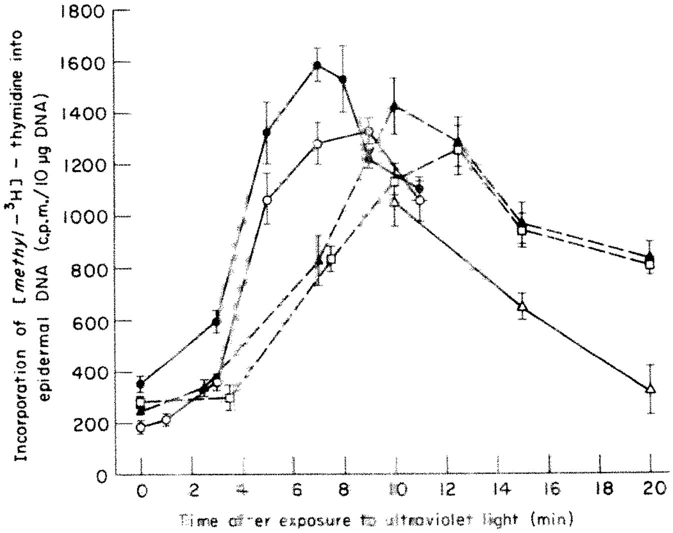

FIGURE 2. Effect of dose of ultraviolet light on the rate of epidermal DNA synthesis. The animals were kept in the environment described in Fig. 1. Forty-eight hours after irradiation at 10^{-4} W/cm^2 (measured using an International Light radiometer, Model IL 500, set at the UV-B range), for the times indicated, the incorporation of [methyl-^{3}H]-thymidine into epidermal DNA was determined. The animals were irradiated while conscious in restraining cages in two separate experiments (▲ - - - ▲, □ - - - □), or they were irradiated under anaesthesia in three separate experiments (●——●, ○——○, △——△). Each point represents the mean value for five animals; the bars represent the standard errors.

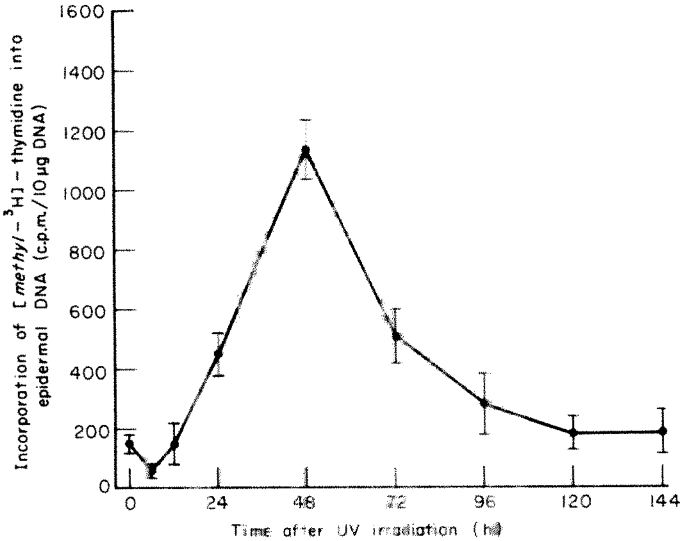

FIGURE 3. Rates of epidermal DNA synthesis at various times after ultraviolet light irradiation. Animals kept in the environment described in Fig. 1 were irradiated for 7 min under anaesthesia. At various times after irradiation, the incorporation of [methyl-^{3}H]-thymidine into epidermal DNA was determined. Each point represents the mean value for five animals; the bars represent the standard errors.

Rates of epidermal DNA synthesis at various times after ultraviolet irradiation

Because the rate of DNA synthesis reportedly increases between 48 and 72 h after UV irradiation (Epstein *et al.*, 1970), in the foregoing experiments we measured the rate of DNA synthesis at 48 h. Nonetheless, we judged that it was important to examine the time course of DNA synthesis under the optimized conditions described above. As shown in Fig. 3, the rate of epidermal DNA synthesis was maximal at 48 h after UV irradiation, and this time was used in all subsequent experiments.

Effect of ambient temperature on the DNA synthesis in the epidermis

From time to time we obtained results from the epidermal hyperproliferation assay in which the rate of DNA synthesis in the non-irradiated controls was 50–100% greater than that usually seen, and the rate of DNA synthesis in the ultraviolet light-irradiated controls was 40–60% less than that normally observed. In such experiments, the difference between the non-irradiated and irradiated controls was often as little as 100–200 c.p.m./10 μg DNA. Because such results often were obtained at times of cold weather, with its accompanying draughts and relative humidity changes, we decided to test the effects of different ambient temperatures on the rate of DNA synthesis in irradiated and non-irradiated epidermis.

The results of one such investigation are shown in Table 1. Clearly, increasing the temperature from 20° to 23·5° resulted in a dramatic improvement in the difference in the apparent rate of DNA synthesis in the epidermis resulting from both a decrease in the rate in the non-irradiated animals and increase in the rate in irradiated animals. A further increase in the temperature (from 23·5° to 27°) resulted in an additional improvement in the difference between the irradiated and non-irradiated rates of DNA synthesis. At 27°, the rate of DNA synthesis in irradiated epidermis is four times that in non-irradiated epidermis.

TABLE 1. Effect of ambient temperature on DNA synthesis in ultraviolet-irradiated and non-irradiated epidermis

	Incorporation of [methyl-^{3}H]-thymidine into epidermal DNA (c.p.m./10 μg DNA). Animals kept at:		
Treatment	20 ± 0·5°	23·5 ± 0·5°	27 ± 0·5°
UV-irradiated	1006 ± 140	1630 ± 206	1811 ± 322
Non-irradiated	793 ± 93	544 ± 35	436 ± 51
Difference	213 ± 233	1086 ± 241	1375 ± 373

Animals were kept in the environment described in Fig. 1 at the ambient temperature indicated and irradiated for 7 min under anaesthesia. Forty-eight hours after ultraviolet irradiation, the incorporation of [methyl-^{3}H]-thymidine into epidermal DNA was determined (see Methods). The values presented are the means for five animals ± standard error.

Now that we are aware of the profound effect of the ambient temperature on epidermal hyperproliferation assay, we are taking special care to ensure that the animals are maintained at 27 ± 0·5° and are kept away from draughts.

DISCUSSION

Because there is no known naturally occurring disease analogous to psoriasis in animals, those searching for new antipsoriatic drugs have had to rely on psoriasis-testing models in animals. Although it has not yet been possible to induce the disease in animals, one of the primary properties of the disease 'epidermal hyperplasia' can be readily produced in animals by a variety of means. The prevention of such induced epidermal hyperplasia is taken as an indication that the inhibitory agent may be useful for the treatment of psoriasis.

The hairless mouse has been useful in the search for new topical and systemic therapies for psoriasis (Prunieras, 1981). The investigations summarized here should help other investigators to set up the hairless mouse screening model in their laboratories. All the factors investigated have a significant effect on the usefulness of the assay. The diurnal variation in the non-irradiated skin may depend on the timing of light-dark cycles to which the animals are subjected. DNA synthesis decreased steadily for the first 6 h of the light cycle and then began a slow increase. These data are in agreement with those of others who studied the diurnal fluctuation in epidermal cyclic AMP (Marks & Grimm, 1972) which has been shown to inhibit epidermal mitotic activity in a dose-dependent manner (Voorhees *et al.*, 1974). Investigators who house their animals under a different light-dark cycle from that used here would be well advised to re-examine the diurnal variation in epidermal DNA synthesis in their animals as a first step to setting up the hairless mouse screening model.

The light dose used to produce epidermal hyperproliferation must be carefully controlled to achieve the maximal DNA synthesis. If the time of ultraviolet light exposure is too short, a less than maximal rate of DNA synthesis is achieved. Likewise, if the time of exposure is too long, the rate of DNA synthesis is also less than that maximally obtainable. That this was due to damage to the epidermis that was apparently not repairable in 48 h was confirmed by histological examination of the epidermis. It is important to note that the outputs of commercially available ultraviolet sunlamps are variable; hence, for each set of lamps used, the minimum dose of ultraviolet light needed to produce the maximal DNA synthesis must be determined. In addition, we have found that the field of light under a bank of ultraviolet sunlamps does not have the same energy in all parts of the field. As would be expected, the centre of the field has more energy than the edges. Therefore, because mice are usually irradiated in groups under the same bank of lamps, it is important to verify that all animals are receiving the same dose of radiation.

An unexpected observation was the dramatic effect of ambient temperature on the rate of DNA synthesis in both irradiated and non-irradiated epidermis. Although the data presented here do not explain this phenomenon, two possibilities deserve mention. Because they are hairless, the mice may respond to cold by a generalized increase in the metabolic activity of the epidermis, which results in an increase in the rate of DNA synthesis in non-irradiated epidermis. The decreased response at colder temperatures of the ultraviolet light-induced increase in DNA synthesis may be a response to cold stress. Stress would be expected to result in an increase in plasma catecholamines and/or glucocorticoids, both of which are known to have an antimitotic effect on the epidermis (Voorhees *et al.*, 1972; Goodwin & Fry, 1976).

ACKNOWLEDGMENT

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The influence of treatment on fibrin microclot generation in psoriasis

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SUMMARY

The *in vitro* formation of fibrin microclots in blood from patients with psoriasis was studied before, during and after treatment. When a bacterial lipopolysaccharide was added to the blood, the microclot test was positive in 0% of controls, in 17% of the patients with untreated slight psoriasis and in 100% of those with severe psoriasis. A positive test without added lipopolysaccharide indicates the presence of circulating endotoxins. It was negative in all patients with slight psoriasis but positive in 75% of those with a severe form.

During treatment the microclots decreased concurrently with clinical improvement in 79% of the patients. After treatment, eleven patients still had a positive test and nine of these patients showed a relapse within 1–2 months. Warfarin treatment rapidly inhibited microclot formation but this had little or no effect on the psoriatic lesions. High doses of potent corticosteroids under occlusion inhibited microclot formation for some hours.

It seems likely that there may be a release of endotoxins in severe psoriasis which is decreased during successful treatment.

In patients with psoriasis and certain other disorders microclots can be induced by incubating the buffy coat from blood samples with bacterial lipopolysaccharides (Juhlin & Shelley, 1977). The microclots appear as a radiating fibrin crystallization which usually has a monocyte in the centre. The phenomenon is not seen in blood from healthy subjects. In certain disorders such as severe psoriasis, eczema, chronic urticaria, ulcerative colitis, Crohn's disease and cancer of the gastro-intestinal tract, the microclots may be generated without addition of bacterial lipopolysaccharides (Juhlin & Shelley, 1981). The microclots in these diseases seem to reflect the presence of circulating endotoxins. Since the ability to produce microclot formation could be important for the development of psoriatic lesions we have investigated the value of the tests in patients with psoriasis of varying severity. Patients with severe psoriasis were followed before, during and after treatment with PUVA, retinoids or local treatment. The effect of oral warfarin on psoriatic lesions was also tested since it can block microclot formation.

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METHODS

Ninety patients, aged 19–68 years, with classical psoriasis were studied. The degree of severity was graded I to IV.

Grade I: a few psoriatic plaques with a total surface of 1–3 dm^2.
Grade II: plaques of 3–8 dm^2.
Grade III: total psoriatic area of 8–24 dm^2.
Grade IV: extensive psoriasis over 24 dm^2 to total erythroderma.

Patients with psoriasis grade I–II were treated with local corticosteroids or anthralin and UV-irradiation as outlined by Ingram (1953). Those with more severe psoriasis were treated with psoralen bath + UV-irradiation (Fischer & Alsins, 1976), etretinate (Tigason®) 25–30 mg daily often combined with UV-irradiation, methotrexate (10–25 mg weekly), or the Ingram regimen. The effect of potent corticosteroid ointments (fluocinolone, betamethasone or clobetasol) 15–20 g under occlusive dressing on large areas for 4 h on one occasion was tested in eight patients. Warfarin (Waran®) was tried in ten patients with severe psoriasis where other treatments had failed. The dose was adjusted according to the prothrombin index which was lowered to 20% or more.

The fibrin microclot formation was studied in microcapillary tubes after adding an endotoxin lipopolysaccharide type B (*E. coli* 0128:B12 Difco, Detroit, Mich.) to buffy coat suspended in plasma as described by Juhlin & Shelley (1977). The test was considered as positive when more than six stellate fibrin microclots were formed in a capillary tube after 24 h.

RESULTS

The fibrin microclot test after addition of endotoxin was positive in 17% of the patients with slight psoriasis and in 100% of those with very severe psoriasis (Table 1). Without addition of endotoxin the test was negative in patients with slight psoriasis but positive in 75% of the patients with severe psoriasis

In thirty-four patients with psoriasis grade III–IV (Table 2), the microclot test with added endotoxin was performed before and during treatment. In patients showing clinical improvement a simultaneous decrease in the number of microclots was noted in seventeen patients. The microclots remained unchanged in ten patients who did not improve and increased in three

TABLE 1. The results of the fibrin microclot test in seventy-one patients related to severity of psoriasis. Figures show number of patients

	Results of fibrin microclot test	Severity grade of psoriasis			
		I	II	III	IV
Endotoxin added	Pos.	2	11	20	12
	Neg.	10	14	2	0
Endotoxin not added	Pos.	0	4	16	9
	Neg.	12	21	6	3

TABLE 2. Effect of treatment on clinical condition and fibrin microclot test with addition of endotoxin

Treatment	Number of patients	Change in psoriasis	Change in fibrin test
PUVA bath	8	Improvement	Decrease
PUVA bath + retinoid	2	Improvement	Decrease
Ingram	3	Improvement	Decrease
Methotrexate	1	Improvement	Decrease
PUVA bath	5	Improvement	No change
PUVA bath + etretinate	2	Improvement	No change
PUVA bath + etretinate	10	No change	No change
PUVA bath + etretinate	2*	Worse	Increase
Etretinate	1*	Worse	Increase

* Later the number of fibrin microclots decreased and their condition improved.

patients who became temporarily worse (Table 2). In seven patients there was clinical improvement but the fibrin microclot test remained positive.

In some patients, variation in the test result from marked microclot formation to a negative test or vice versa was sometimes seen from one week to another, but a test repeated on the same day or the following day usually gave the same result. Patients who repeatedly had a negative test when no endotoxin was added could sometimes be positive. There was no definite correlation with infection or other disease.

In ten patients with severe psoriasis and no or poor response to earlier treatments, warfarin was given for 4–6 weeks. Local treatment with anthralin and/or tar and UV-irradiation was continued as before. The fibrin test became negative within 24 h, even before the prothrombin time had decreased, and it remained negative during the warfarin treatment. Marked improvement was seen in one patient, but little or no effect was seen in the others.

The effect of a potent corticosteroid under occlusive dressing was tested in eight patients. A decrease in microclot formation was seen after 2–4 h in five patients and there was no change in three patients. In those treated with 2–4 ml of corticosteroids without occlusion no change occurred.

At the end of treatment all patients with an initially negative fibrin test had improved, whereas the test was positive in ten patients who showed no clinical improvement (Table 3). Relapses

TABLE 3. Clinical effect at the end of treatment and result of the fibrin microclot test with added endotoxin. Figures show the number of patients

Fibrin test	Improved	Not improved
Positive	11	10
Negative	14	0

were seen within 1–2 months in nine of eleven patients showing improvement but still having a positive fibrin test.

DISCUSSION

The fibrin microclot formation after adding endotoxin to blood from patients with psoriasis was most marked in those with severe psoriasis. During treatment, a correlation between the change in the patients' condition and the microclot test was seen in 79% of the patients. After treatment, the reaction to endotoxin remained positive in eleven of the patients (32%). In nine of the eleven a relapse occurred within 1–2 months. These findings favour the idea that the fibrin microclots could be associated with the appearance of the lesions. They might also be relevant to the possible increased incidence of occlusive vascular disease in patients with psoriasis (McDonald & Calabresi, 1978). The complete disappearance of fibrin microclots after warfarin treatment without any clinical improvement in most patients indicates that the microclots themselves are not responsible for the persistence of the lesions. It should be stressed, though, that the patients selected for warfarin therapy had severe psoriasis responding poorly to most given treatments. Two patients with generalized but less severe psoriasis improved completely on two occasions after treatment with warfarin for myocardial infarction. Heparin has also been reported to have a favourable effect on two patients with psoriasis (Bauer, 1981).

The effect of endotoxin and various other factors on intravascular coagulation and fibrin formation was reviewed by Müller-Berghaus & Lasch (1975). The occurrence of microclots without adding bacterial lipopolysaccharides indicates an endogenous release of endotoxin (Juhlin & Shelley, 1981). In more severe psoriasis, circulating endotoxins seem to occur frequently. In patients not generating microclots initially, repeated tests over several days showed that a temporary release of endotoxins can occur. This was not observed in healthy subjects. The endotoxins are known to change the reactivity of vascular mediators such as noradrenaline, and they also change the pattern of prostaglandin release (Feuerstein & Ramwell, 1981). They might thus induce vascular changes promoting the development of psoriatic lesions.

Whether the various treatments given directly, like warfarin, can inhibit the microclot formation is uncertain. The microclots disappear rapidly after systemic treatment with corticosteroids (Juhlin, Shelley & Vahlquist, 1979). Potent local corticosteroids at high doses under occlusion have not been found to have a similar effect. Local corticosteroid treatment with lower doses and without occlusion had no effect on the microclots and cannot explain their decrease during treatment. UV-A irradiation of blood with psoralens added has been shown *in vitro* to decrease the number of microclots (Juhlin & Shelley, 1978), but the PUVA bath treatment reported here did not always decrease the microclot test. Treatment with methotrexate or etretinate showed no immediate effect on microclot formation although methotrexate has been reported to decrease coagulation and increase fibrinolysis (Mansurov, 1980).

Is the fibrin microclot test of any value in clinical practice? It indicates a severe type of psoriasis and when it remains positive after treatment the patients seem to relapse earlier. However, since some patients with severe psoriasis had a negative test and some with a positive test improved, its clinical value must be regarded as limited.

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Correlation of epidermal plasminogen activator activity with disease activity in psoriasis

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SUMMARY

The specific activity of plasminogen activator was increased in clinically involved psoriatic epidermis compared with the uninvolved skin of the same eight patients. Alterations in plasminogen activator activity correlated with disease activity as measured by extent of body surface involved with psoriasis. Levels of plasminogen activator increased in uninvolved epidermis of psoriatic patients during retinoid therapy.

Proteinase and exopeptidase activity are increased in rapidly proliferating psoriatic epidermis (Stuttgen & Wurdeman, 1959; Steigleder & Enders, 1964; Schwartze & Hanson, 1971; Mier & Van den Hurk, 1976; Fraki, Lazarus & Hopsu-Havu, 1982b; Lazarus & Fraki, 1981). Proteinases distinct from proteinases found in normal human skin have been demonstrated in psoriatic scales (Fraki & Hopsu-Havu, 1976a, b). A chemo-attractant factor which appears to be a serine proteinase is increased in clinically involved psoriatic epidermis (Lazarus, Yost & Thomas, 1977). Plasminogen activator is a specific proteolytic enzyme which is increased in transformed and activated cells (Reich, 1975; Rifkin, Beal & Reich, 1975). It has been shown that this enzyme is increased in psoriatic scale (Piper, Hadlich & Wurbach, 1967; Haustein, 1969; Haustein, 1971; Fraki & Hopsu-Havu, 1976a, b; Fraki, Djupsund & Hopsu-Havu, 1978) and separated psoriatic epidermis (Fraki *et al.*, 1981; Fraki, Briggaman & Lazarus, 1982a). Recent experiments demonstrate that psoriatic epidermis maintains its increase in specific activity of plasminogen activator even after grafting on to athymic nude mice (Fraki *et al.*, 1981; 1982a). Uninvolved psoriatic skin has normal levels of plasminogen activator. When this tissue is transplanted to the nude mouse for 6 weeks, there is an increase in the specific activity of

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plasminogen activator to the levels observed in clinically involved psoriatic epidermis after transplantation. Increased enzyme levels of uninvolved skin after grafting are associated with an increase in labelling index. These experiments suggest that the increase of plasminogen activator activity in psoriasis is a marker of cell activation and that the elevations of plasminogen activator occur within the skin itself and do not require interaction with circulating factors in the psoriatic host.

Etretinate (RO-10-9359) is an aromatic retinoid which has been shown to help in the treatment of psoriasis (Goerz & Orfanos, 1978). A double-blind controlled study of this therapeutic agent provided an opportunity to study the correlation of plasminogen activator with disease activity in psoriasis.

METHODS

We studied eight patients (aged 20 years or more) who had more than 20% of their skin involved with psoriasis. All topical and systemic therapy was discontinued 1 week before entering the study. Artificial ultraviolet radiation therapy and excessive sun exposure were avoided during the study. At entry into the study a careful estimate of the percentage of the skin surface involved with psoriasis was obtained. Individual plaques were also rated as to amount of scale, erythema and thickness; each of these characteristics was graded on an arbitrary 0–4 scale. The patients were then treated in a double-blind fashion with placebo or etretinate at 1 mg/kg per day in two divided doses. The duration of therapy was 16 weeks followed by an 8-week observation period without treatment. At the 8th week, or if the patient became definitely worse before 8 weeks, the code was broken and patients on placebo were then given the 16-week course of etretinate. The extent of disease was very carefully determined at the 16th week when the drug was discontinued.

Six-millimetre punch biopsies were taken from involved and uninvolved skin after xylocaine anaesthesia before entry into the study and 4, 8 and 16 weeks after beginning the drug. Whenever possible, skin specimens were taken from the hip and proximal thigh and informed consent was obtained from all donors. All specimens were coded and no clinical information was supplied to the laboratory.

Each skin specimen was incubated in 2M potassium bromide (37°C, 30 min) which permits mechanical separation of epidermis from dermis. The harvested epidermis was washed in sterile saline and then frozen in 1 mg/10 μl of 2M KCl/0·01 M Na-phosphate buffer pH 7·0 at −70°C until used. The brief potassium bromide treatment did not affect the level of plasminogen activator (Fraki *et al.*, 1981; Fraki *et al.*, 1982a).

Plasminogen activator activity was measured using a modification of the ^{125}I-fibrinogen method (Berger, Fleming & Voorhees, 1977). Briefly, purified dog plasminogen was used as substrate for plasminogen activator and activated plasmin was quantitated by measuring release of radioactive peptides from ^{125}I-labelled fibrinogen attached to tissue culture plates (Linbro®, Linbro Division, Flow Laboratories, Hamden, Conn.). Bovine fibrinogen was purified (Laki, 1951) from a crude fibrinogen preparation obtained from Calbiochem, and it was labelled with ^{125}I using a BioRad iodination kit. Appropriate controls without plasminogen or sample were used and all assays were performed in the linear dose range of the assay. Protein concentration was measured by the bromsulphalein binding assay (McGuire, Taylor & Greene, 1977) with bovine serum albumem (Sigma) as standard. The DNA content of the epidermis was estimated according to the method of Labarca and Paigen (1980). All enzyme activities are expressed as hydrolytic rate of substrate per quantity of DNA in the sample. Plasminogen activator levels in the samples were quantitated as International Units by comparing fibrinogen hydrolysis produced by the sample with a standard linear dose–response curve of degradation of fibrinogen by Abbokinase. To minimize variation in the plasminogen activator assay, all samples were assayed simultaneously.

Data were analysed using the Wilcoxon signed rank method of non-parametric analysis.

RESULTS

Biopsies of involved and uninvolved skin were obtained from twelve patients before beginning therapy. The specific activity of plasminogen activator was 19-fold higher in involved psoriatic epidermis than uninvolved skin (two-sided P value < 0·0037). These data confirm our previous observations (Fraki *et al.*, 1981; 1982a). Biopsies of involved and uninvolved skin were also obtained from patients after 8 weeks of therapy. The specific activity of plasminogen activator in involved skin after 8 weeks of therapy was correlated with the extent of surface involvement with psoriasis at 16 weeks. This correlation was made because the most dramatic alterations in extent of disease were obtained at 16 weeks (Fig. 1).

Eight patients were studied. Six patients had a decrease in the extent of cutaneous involvement at 16 weeks, and five of these had decreases in plasminogen activator activity in their epidermis when compared to pre-therapy levels. One patient had a modest diminution in extent of skin involvement but his plasminogen activator activity increased slightly. Two patients had an increase in extent of skin involvement after 16 weeks of therapy; both of these

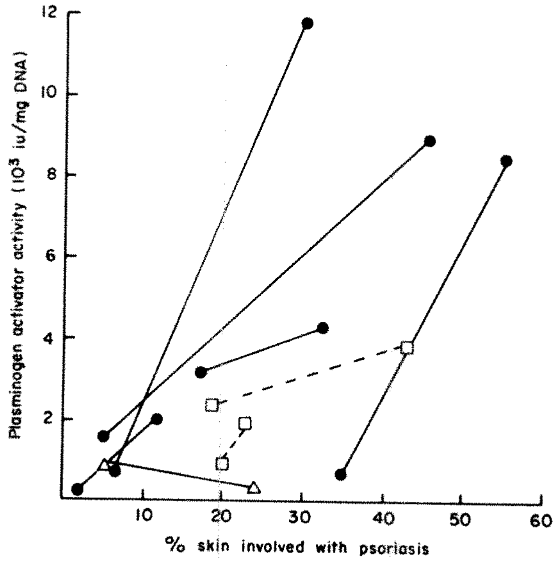

FIGURE 1. Plasminogen activator activity (units/mg DNA) plotted against extent of psoriasis. Values observed before beginning therapy are compared with values obtained at 8 weeks (for plasminogen activator) and 16 weeks (for extent of psoriasis) while on retinoid. Each pair of points represents a single patient. ●——● Five patients who had a decrease in plasminogen activator activity at 8 weeks and a decrease in extent of psoriasis at 16 weeks. □——□ Two patients who had increased levels of plasminogen activator at 8 weeks and increased extent of psoriasis at 16 weeks. △——△ One patient who had a decrease in skin involvement at 16 weeks, but had a modest increase in plasminogen activator activity level at 8 weeks.

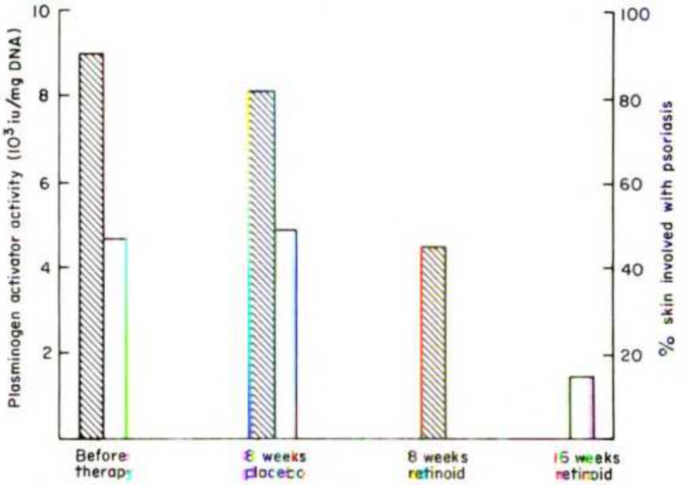

FIGURE 2. Plasminogen activator activity and extent of psoriasis in a single patient before and after treatment. ▨ Plasminogen activator activity. ☐ Percentage skin involved with psoriasis.

patients had increases in plasminogen activator activity in involved epidermis at 8 weeks. When the slopes of these lines (Fig. 1) were analysed using the Wilcoxon signed rank method, there was a significant correlation between extent of disease and plasminogen activator activity (two-sided P value $< 0{\cdot}017$).

Figure 2 presents the data from a single patient. There was no change in extent of disease of plasminogen activator activity while the patient was on placebo. Eight weeks after beginning retinoid the plasminogen activator level fell, and by 16 weeks there was a dramatic decrease in the extent of the patient's psoriasis.

The plasminogen activator levels of uninvolved skin specimens which had low specific activities of enzyme before beginning therapy rose at 8 weeks in five patients. By contrast, two patients who initially had high plasminogen activator levels had normal values at 8 weeks, and in both patients there was a significant decrease in the extent of skin disease at 16 weeks.

DISCUSSION

There is a dramatic increase in the specific activity of plasminogen activator in psoriatic skin when compared to clinically uninvolved skin. There was some correlation between the specific activity of plasminogen in the involved psoriatic plaque and the extent of skin involved upon entry into this study. Unfortunately, our numbers were small and the degree of involvement of our patients was similar; consequently, no statistically significant correlation could be obtained. There was no correlation of plasminogen activator with the clinical type of psoriasis. It is unlikely that the activity which we measured in psoriatic epidermis was a consequence of contamination by fibrinolytic skin surface lipids (Dawber, Nishioka & Ryan, 1971) since our activator was a serine proteinase. Normal human skin contains inhibitors of plasminogen activator (Ryan, Nishioka & Dawber, 1971; Fraki *et al.*, 1982a; Hashimoto *et al.*, 1982). Undoubtedly, the activity measured is a consequence of the dynamic balance between enzyme and inhibitor.

The specific activity of plasminogen activator in involved epidermis at 8 weeks was correlated with extent of skin involved with psoriasis at 16 weeks. This correlation was selected since the most dramatic alterations in extent of disease appeared between 8 and 16 weeks. It was of interest to investigate the plasminogen activator levels immediately before the time of most dramatic clinical change. Six patients had significant diminution in the extent of skin disease at 16 weeks. In five patients the specific activity of plasminogen activator at 8 weeks decreased from the levels before beginning therapy. One patient with clinical improvement had a slight increase in plasminogen activator at 8 weeks. There was nothing clinically unique about this patient. Two patients had an increase in extent of psoriasis while on retinoid. Both of these patients had increases in the specific activity of plasminogen activator in involved skin at 8 weeks when compared to levels before beginning therapy. These data indicate that increases in the specific activity of plasminogen activator may correlate with disease activity as measured by extent of skin involvement. We were unable to establish a correlation of plasminogen activator with the severity of individual lesions. This may reflect our inability to precisely quantitate alterations in a specific psoriatic lesion.

It is unlikely that the alterations in plasminogen activator level were due to the aromatic retinoid. All the patients were taking the drug, and plasminogen activator levels decreased in patients with improvement and increased in patients with progressive disease. Brinkerhoff *et al.* (1980) have shown that 13-*cis*-retinoic acid and all-*trans* retinoic acid diminished collagenase production by adherent rheumatoid synovial cells *in vitro*. Gelatinase and acid proteinase secretion were not affected by the drug and plasminogen activator levels were not reported in their study. Retinoic acid increases plasminogen activator synthesis by normal and malignant cell lines of mesenchymal origin *in vitro* (Wilson & Dowdel, 1980). By contrast, these authors reported that retinoic acid, retinol and retinyl acetate had little effect on normal and neoplastic epithelial cell line production of plasminogen activator *in vitro* (Wilson & Dowdel, 1980). The base line production of plasminogen activator by normal and neoplastic epithelial cell lines was considerably higher than mesenchymal cells. The effect of the aromatic retinoid on plasminogen activator synthesis in normal human epidermal cells in culture is presently under study.

Our data suggests that plasminogen activator activity in psoriatic plaques is a good marker of cellular activation in this disease. The correlation of plasminogen activator activity with extent of disease over time in a series of patients on etretinate suggests that plasminogen activator activity may be a useful marker in the study of psoriasis.

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Effects of methotrexate *in vitro* on epidermal cell proliferation

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SUMMARY

Epidermal cell migratory activity and epidermal cell proliferation are clearly dissociable in explant culture. Epidermal cell migration requires a non-dialysable, 65,000 mol.wt factor which is destroyed at 100°C but is stable at 80°C for at least 30 min. In the presence of dialysed serum or heated serum (80°C for 30 min) or DNA synthesis inhibitors (methotrexate or hydroxyurea), cells will migrate from the explant but will not proliferate. At least two factors are required for normal proliferation under these restricted conditions—an adequate supply of DNA precursers, i.e. nucleosides, and a heat labile (80°C) non-dialysable serum component.

Methotrexate in concentrations of 1·0 μg/ml or greater added to cultures in normal fetal calf serum significantly inhibited mitoses; however, when added to serum dialysed to remove thymidine, mitotic inhibition occurred at a concentration of 0·1 μg/ml of methotrexate and when added to dialysed serum and kept in dialysed serum, inhibition occurred with 0·01 μg/ml of methotrexate. Methotrexate did not inhibit outgrowth. Hydroxyurea also inhibited mitoses but did not effect outgrowth.

Considerable information is available about the effect of methotrexate (MTX) *in vivo* on psoriatic epidermal proliferation (Fry & McMinn, 1966; Weinstein, Goldfaden & Frost, 1971; Newburger, Weinstein & McCullough, 1978) but only a few studies have been published reporting the *in vitro* effects of MTX on epidermal proliferation. Karasek (1971) reported that MTX even in concentrations as high as 1000 μg/ml did not inhibit the outgrowth of normal human epidermal explants. Flaxman *et al.* (1977) reported that MTX in concentrations as low as 0·1 μg/ml inhibited mitotic activity in human epidermal explants. Although both Karasek and Flaxman used human epidermal explants in their studies, Karasek measured epidermal outgrowth but did not determine the mitotic index whereas Flaxman determined the mitotic index but did not measure epidermal outgrowth. In this paper we describe the effects of hydroxyurea and MTX *in vitro* on both mitotic activity and epidermal cell outgrowth. Using

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our tissue culture system we can show that epidermal outgrowth is not dependent on mitotic activity and that proliferation and outgrowth size can be studied independently of each other.

METHODS

Explant cultures were obtained from adult domestic pigs. This system was patterned after an organ culture system using mouse epidermis (Halprin, Lueder & Fusenig, 1979) and the important growth determinants and cell cycle kinetics of our adult pig skin organ culture explant system have been previously described (Hammar & Halprin, 1981). These are very similar to the characteristics of adult human skin (Flaxman, Lutzner & VanScott, 1967; Karasek, 1966; Flaxman & Harper, 1975).

Description of the system

Skin was removed from the pig (Fig. 1) with a Castoviejo keratome at a depth of 0·2–0·3 mm and multiple 2 × 2 pieces were cut using a sharp scalpel, taking care that the edges were clean and perpendicular to the surface. The resulting explants were placed on individual glass cover slips, dermis side down, and allowed to dry for 15–20 min to allow adherence before being placed in a 3·5 cm Falcon petri dish and covered with 2 ml of culture medium. The growth medium consists of RPMI 1640 which contains antibiotics (penicillin 10 u/ml, streptomycin 100 μg/ml, fungizone 0·25 μg/ml) to which fetal calf serum (FCS) and/or various test substances are added. Cultures were incubated in a humidified incubator in an atmosphere of 95% air, 5% carbon dioxide.

During the first 24 h the epidermal cells migrated from the original explant down the cut sides of the dermis and attached to the underlying cover slip. After 24–48 h, the epidermal cells which had reached the cover slip formed a monolayer in a ring around the original explant.

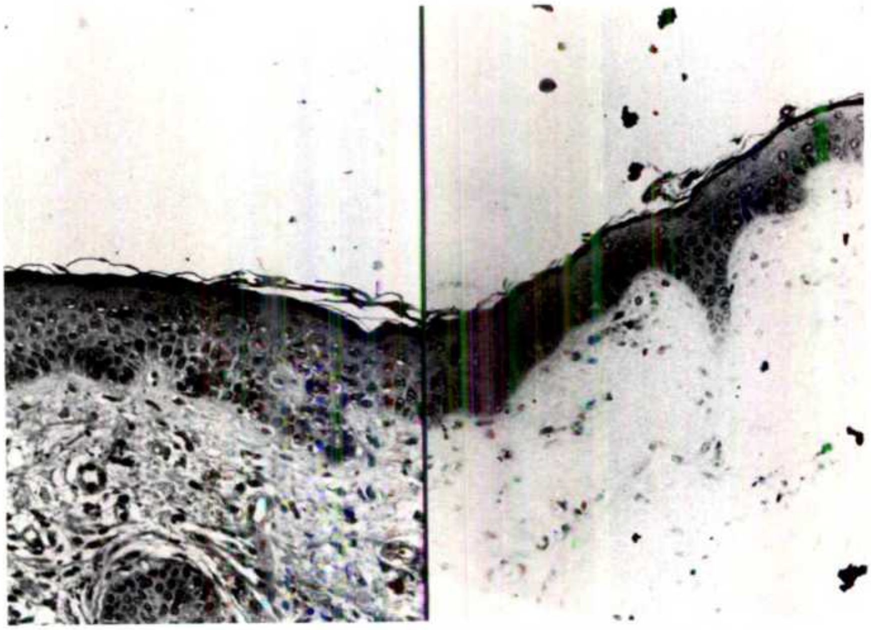


FIGURE 1. Comparison of histology of human (L) and pig (R) skin (× 70).

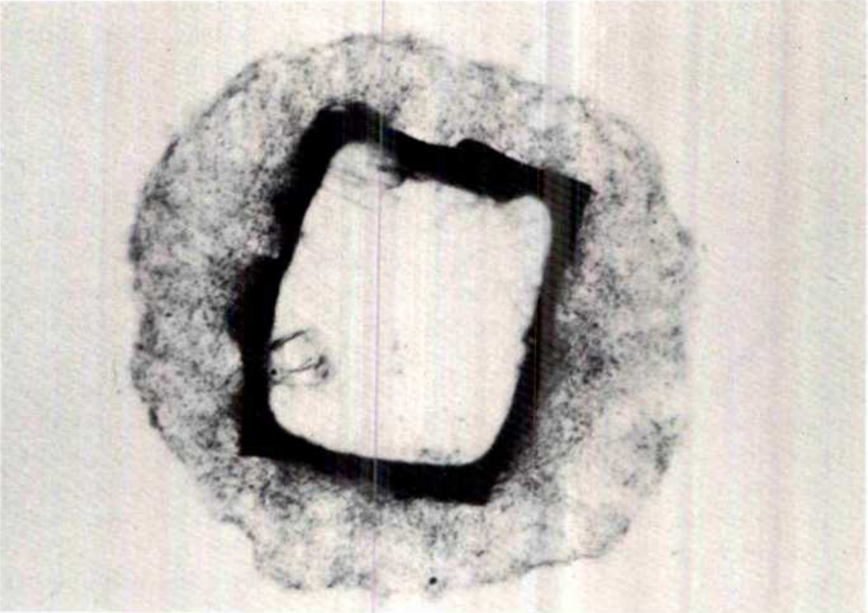


FIGURE 2. Normal explant culture at 96 h (× 14).

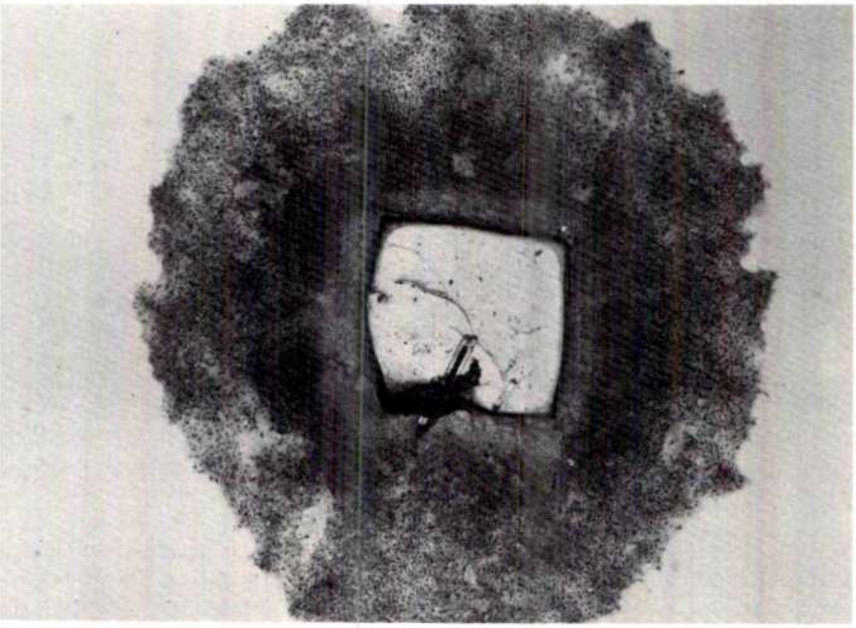


FIGURE 3. Normal explant culture at 6 days (× 70).

Subsequently, in the presence of 10% normal FCS, the cells moved out as a sheet from the explant (Fig. 2) to a distance of 1000 to 2000 μm (Fig. 3) at a speed of approximately 150–250 μm per day (5–10 μm/h). During this process the outgrowth nearest the original explant stratified and differentiated (Fig. 3). There was no need to change the medium during the subsequent 7–10 days.

During the first 48–96 hours, the great majority of epidermal movement was due to epidermal migration without mitotic activity. After this time, most of the enlargement of the epidermal sheet was usually related to epidermal mitotic activity (Hammar & Halprin, 1981) although, as will be shown, this was not always the case. Agents which decrease mitotic activity may not, in some circumstances, decrease the speed with which the epidermal sheet enlarges.

The control culture shown in Fig. 2 at the end of 96 h of culture had four sides growing to a distance of approximately 500 μm and a mitotic index of approximately 5 cells per 1000 cells. During the period from 96 h to 10 days, the epidermis moved out from a distance of 500 μm to approximately 1500 μm and the cell numbers increased from 2000/culture to about 6000/culture. The original explant rate of outgrowth (first 96 h) depended on serum and was directly related to the serum concentration up to a maximum of perhaps 30% serum; that is, the higher the concentration of serum, the faster and larger the outgrowth was during the first 24–96 h. Thereafter, the serum could be changed to lower concentrations without sacrificing the rate of expansion of the outgrowth (Hammar & Halprin, 1981).

A typical experiment is shown in Fig. 4. Eighty explants were attached to cover slips and allowed to grow; of the eighty, seventy-four (92%) grew. When examined at 72 h of growth,

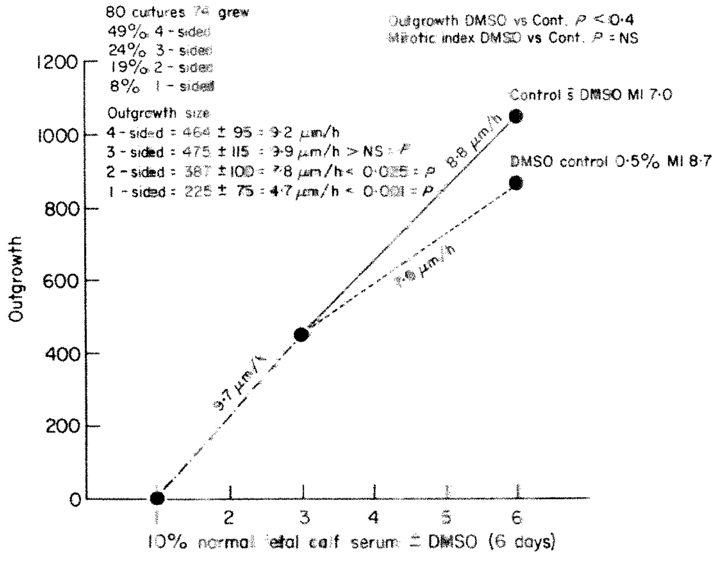


FIGURE 4. Graph of explant outgrowth. Outgrowth was measured by means of an ocular micrometer in the phase contrast microscope on living cultures. Mitotic index (MI) was determined by counting all mitotic figures in the cultures and dividing by the number of cells in the outgrowth. The MI is given as dividing cells per thousand cells. The number of cells in the culture is obtained from measurements of the outgrowth's furthest radius in microns using the formula $\ln N = -5{\cdot}97 + 2{\cdot}78 \ln R - 0{\cdot}1 (\ln R)^2$, where N = number of cells, R = radius of outgrowth (see Hammar & Halprin, 1981). This formula takes into account the fact that the outgrowth is usually not round but ellipsoid. Five cultures for each point were used for the MI. DMSO produced no significant effect on outgrowth.

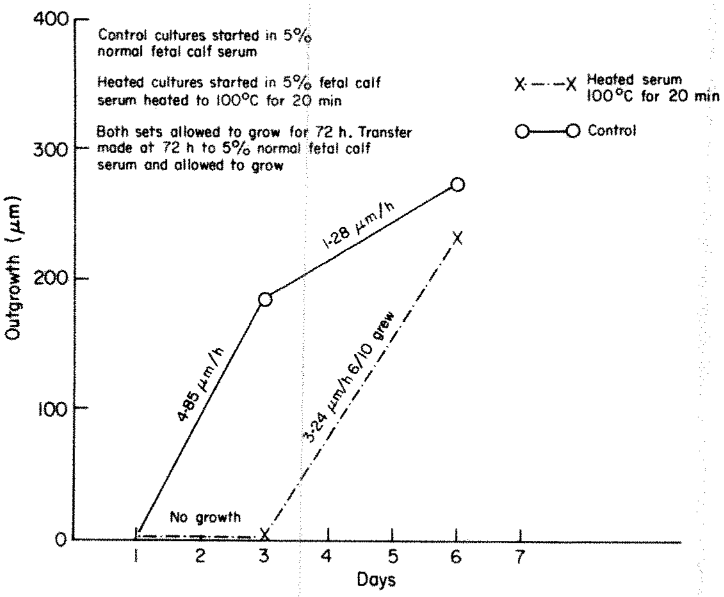


FIGURE 5. Effect of heated fetal calf serum (FCS). Ten cultures were used for each time period. The mean and standard deviation (s.d.) in μm/h for the control cultures was 4·85 ± 1.28. The experimental cultures grew at 3.24 ± 1·36 μm/h.

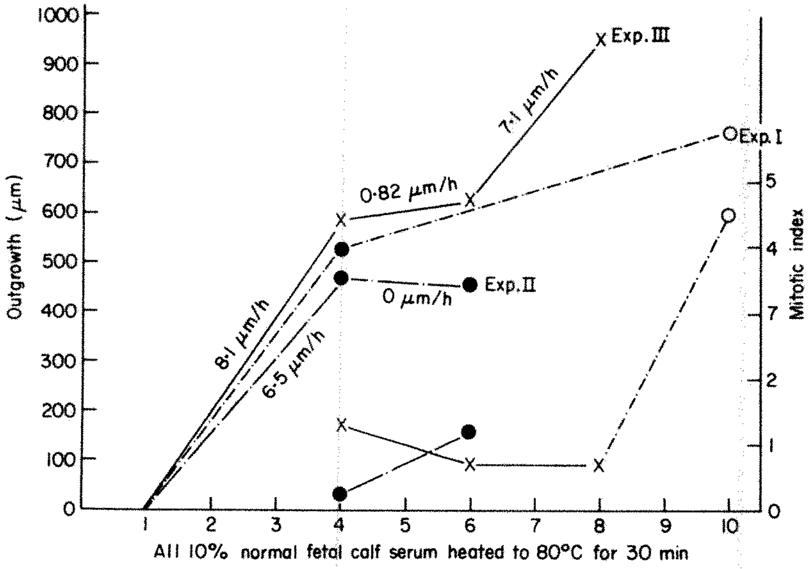


FIGURE 6. Effect of heated FCS (80°C). Each point is the average of five cultures which were removed for outgrowth and MI at the indicated periods. The MI for these cultures was 0·20 ± 0·43 (experiment 2) and 1·29 ± 1·75 (experiment 3) at 4 days of culture; at 6 days the MI and s.d. was 1·16 ± 1·61 (experiment 2) and 0·70 ± 0·91 (experiment 3). At 8 days it was 0·8 ± 1·0 (experiment 3) and at 10 days it was 4·5 ± 3·3 (experiment 1).

49% of the explants had four sides growing, 24% had three sides, 19% had two sides, and 8% had one side growing. Explants which had either three or four sides growing gave essentially the same outgrowth rate of approximately 9·7 μm/h (measurements were started after the first 24 h which is required for the cells to reach the cover slip). The mitotic index for these same explants was approximately 7·0. Explants with only one or two sides growing were depressed significantly and were discarded. Since experiments were started only with explants with three or four sides, the experimental situation was the same for all cultures.

The serum factor required for cells to initially migrate was destroyed by heating the FCS to 100°C for 20 min. Cultures kept in such FCS remained attached but did not show any cell migration onto the cover slip. However, if after 4 days FCS was added that had not been heated, the cultures started to grow (Fig. 5). If the FCS was heated to 80°C for 30 min, explants grew out slightly smaller than their normal counterparts, but they had a much reduced mitotic index at the end of 96 h (Fig. 6). The initial phase of migration was essentially unchanged by this treatment. It was therefore possible to destroy the proliferation factor in the FCS at 80°C while leaving intact the epidermal migration factor. If the cultures were left for 10 days, mitotic activity reappeared (Fig. 6).

Figure 7 demonstrates that the mitotic activity could be restored by adding normal (non-heated) FCS. Within 24–72 h there was a sharp peak of mitotic activity. The cultures were highly synchronous. Presumably the cells were collected in a particular part of the cell cycle (G_1?) in the absence of the heat labile factor necessary for cell proliferation.

Using this tissue culture system, we were able to control cell proliferation while maintaining normal migration by destroying a heat labile proliferation factor. Since migration and proliferation are clearly dissociable, it was possible to measure the effects of MTX and hydroxyurea by adding various concentrations of these substances to the growth medium or FCS and determining mitotic index and migration rate at various times.

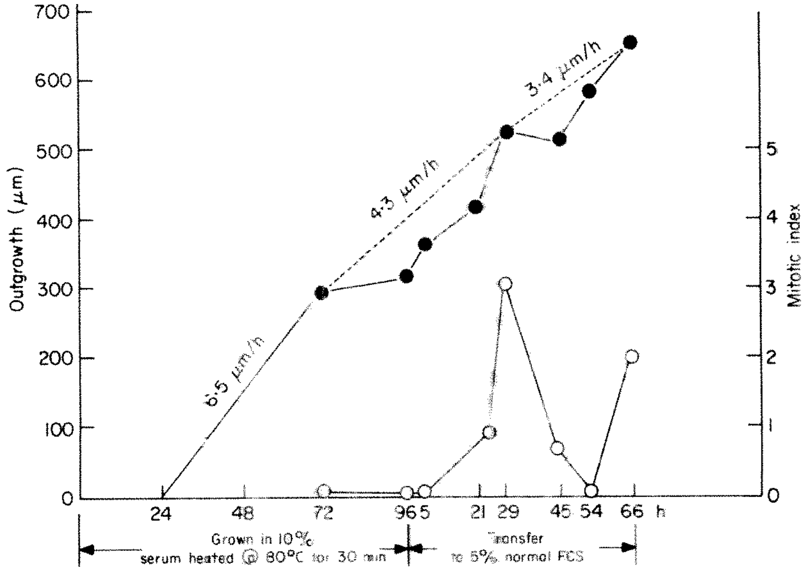


FIGURE 7. Effect of transferring to normal FCS cultures started in heated FCS. Each point is the average of five cultures. The mean and s.d. of the MI for each time period was: 72 h, 0; 96 h, 0; 5 h after transfer, 0·2 ± 0·45; 21 h after transfer, 0·95 ± 0·9; 29 h, 3·07 ± 1·4; 45 h, 0·7 ± 1·05; 54 h, 0·1 ± 0·2; 66 h, 2·0 ± 1·6.

RESULTS

Effects of hydroxyurea on the system

Hydroxyurea added to growing cultures will completely inhibit mitotic activity (data not shown). Figures 8, 9 and 10 show hydroxyurea added to culture medium at the beginning and washed out after 96 h of culture. Hydroxyurea (10^{-2} M) inhibited mitosis completely during the first 96 h; however, it had no effect on outgrowth size. After removal of the hydroxyurea at 96 h, there was still no significant differences between control (3·6 μm/h) and the hydroxyurea treated cultures (2·4 μm/h) 72 h after washout. Mitotic activity was completely inhibited and did not return after washout of the hydroxyurea. The control cultures showed some synchrony of mitoses with a cell cycle time of approximately 68 h.

Figure 9 demonstrates the effect of hydroxyurea histologically at 4 days; Fig. 9a is the control and Fig. 9b is hydroxyurea-treated. The hydroxyurea-treated culture consists of one layer of stretched cells (Fig. 9c); there was no stratification of cells to form multilayers as in the control. The cells in the hydroxyurea-treated culture spread in a monolayer and developed large gaps between groups of cells.

Hydroxyurea at 10^{-3} M was also tested (Fig. 10) and it did not affect outgrowth size but did completely inhibit mitoses during the first 96 h. After washout the cultures gradually recovered mitotic activity; by 3 days after washout the mitotic activity was almost back to normal.

Effect of methotrexate on the system

MTX produced effects similar to hydroxyurea; mitotic activity was depressed while epidermal

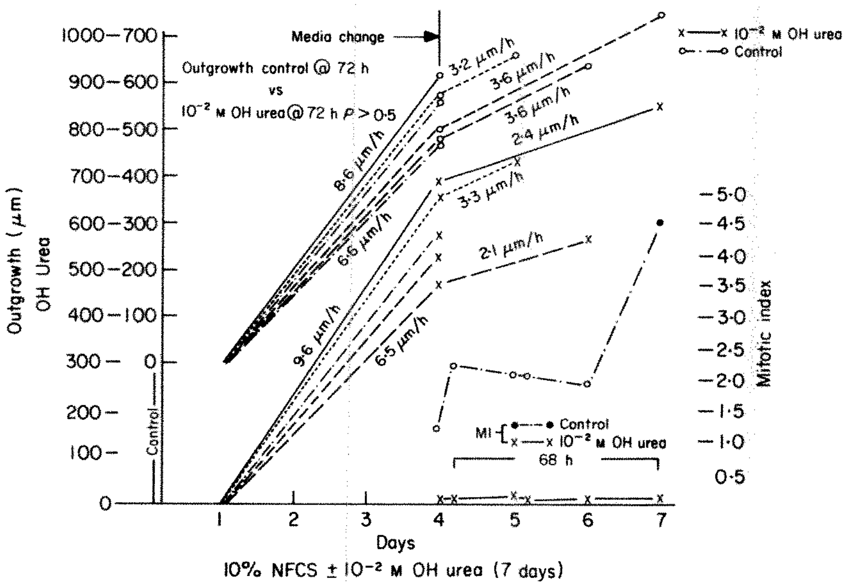


FIGURE 8. Effect of hydroxyurea 10^{-2} M. Five cultures for each group. The outgrowth for each culture group is shown. There was no difference between control and hydroxyurea-treated cultures (note the baseline shift for control cultures). MI of the control was: 1·2 ± 0·6 at 96 h before transfer; 4 h after transfer, 2·3 ± 0·9; 24 h, 2·1 ± 0·9; 28 h, 2·0 ± 0·7; 48 h, 1·9 ± 0·8; 72 h, 4·6 ± 1·4. Hydroxyurea-treated cultures were: 0 at 96 h before transfer; 4 h after transfer, 0; 24 h, 0·05 ± 0·1; 28 h, 0; 48 h, 0; 72 h, 0·07 ± 0·2.

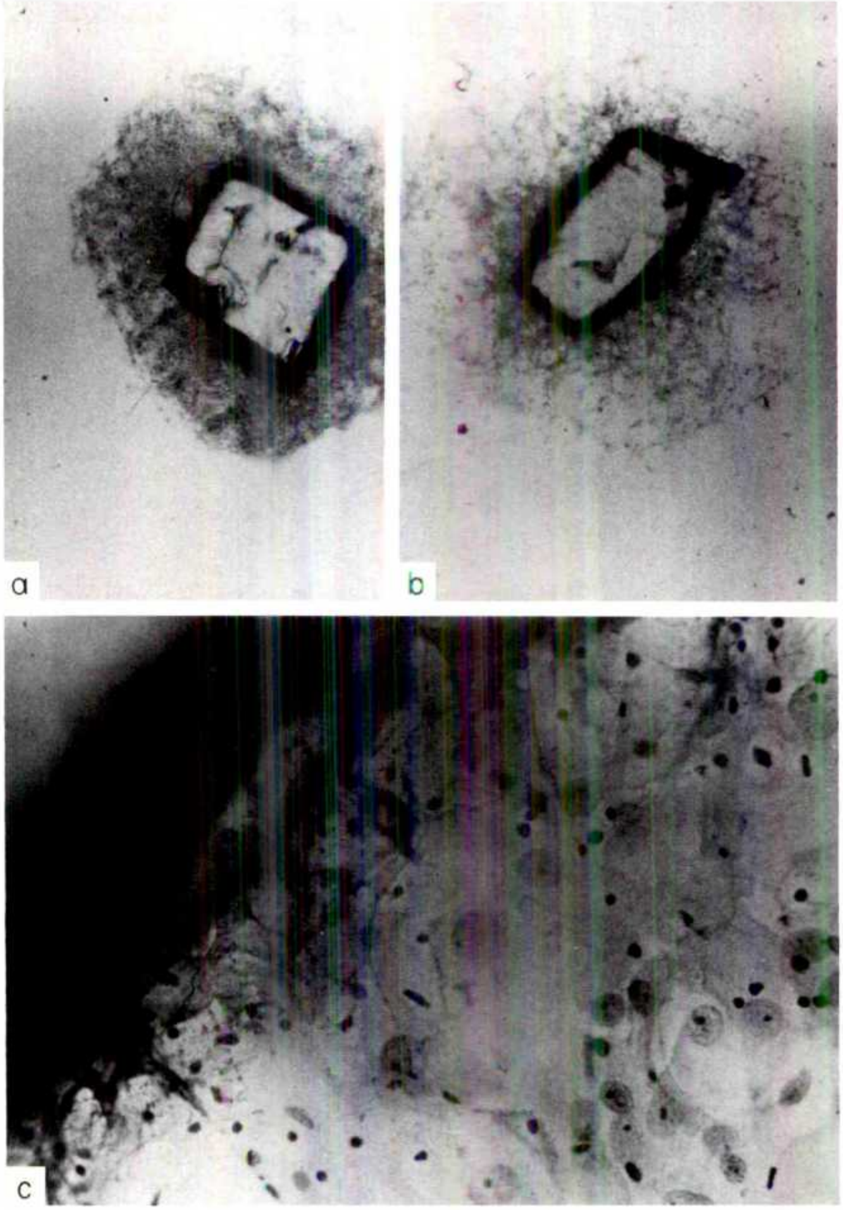


FIGURE 9. Effect of hydroxyurea 10^{-2} M. (a) Normal culture at 4 days (× 70). (b) Hydroxyurea-treated culture at 4 days (× 70). (c) Hydroxyurea-treated culture at 4 days (× 700).

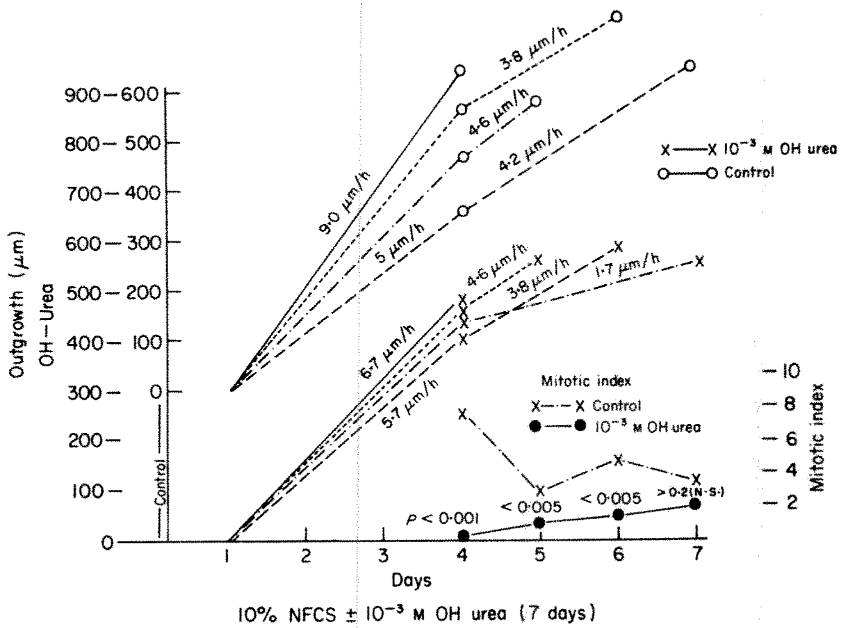


FIGURE 10. Effect of hydroxyurea 10^{-3} M. Five cultures for each group. There is no effect on outgrowth. MI and s.d. of the controls was: at 96 h, 7·7 ± 3·4; after transfer at 24 h, 2·8 ± 0·4; 48 h, 4·8 ± 1·7; 72 h, 3·4 ± 1·7. Hydroxyurea: at 96 h, 0; after transfer at 24 h, 1·0 ± 0·8; 48 h, 1·3 ± 1·0; 72 h, 1·8 ± 1·6.

migration was not significantly inhibited. MTX (1 mg/ml) added to growing explant cultures in 10% normal FCS inhibited outgrowth by only 22% and mitotic activity by 50–75% after 72 h (Fig. 11).

In a separate experiment colchicine was added to determine whether the mitotic activity had

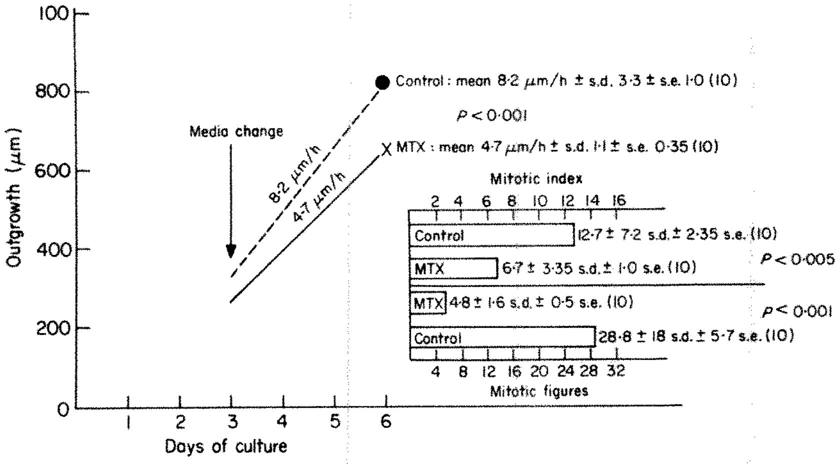


FIGURE 11. Effect of methotrexate (MTX) 1 mg/ml. Cultures were started in 10% FCS and grown for 3 days; ten each were then transferred to either 10% FCS or 10% FCS containing 1 mg/ml MTX. After 3 days the effect on outgrowth, MI and the number of mitotic figures per culture was determined and is indicated. MTX inhibited outgrowth by 20% and mitotic activity by 50–75%.

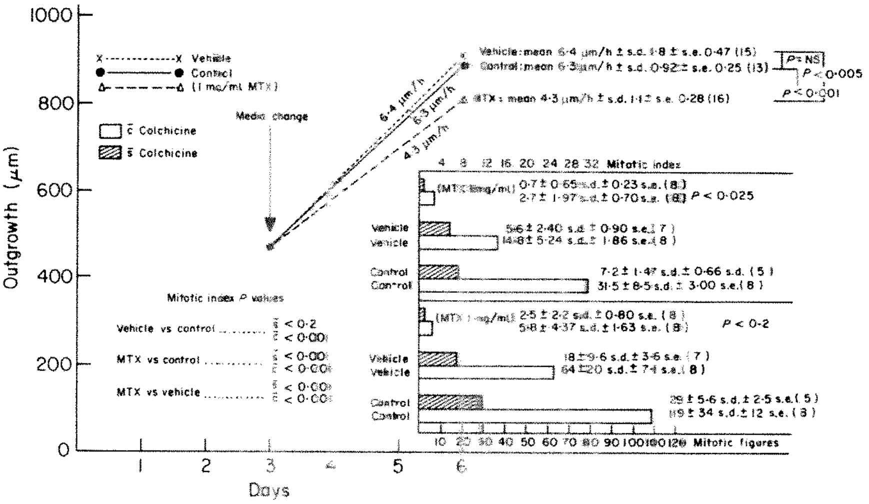


FIGURE 12. Effect of colchicine on continued mitotic activity in the presence of MTX. This experiment is similar to the one in Fig. 11 except colchicine was also added (0·075 mg/ml) for the final 4 h of culture to determine if mitoses continued to occur after the addition of MTX (1 mg/ml). MTX vehicle was included as a second control. (Vehicle contains 0·9% benzyl alcohol, 0·26% NaCl, 40 μl/ml added.) Outgrowth rate, MI and mitotic figures/culture are indicated with s.d. standard error (s.e.), number of cultures in parentheses and *P* values.

begun prior to the MTX inhibition. Since new mitotic figures accumulated during a 4-hour period, this possibility was ruled out (Fig. 12). There was slight but significant inhibition of outgrowth size. Inhibition of mitoses was 5-fold by mitotic index and 10-fold by counting mitotic figures per culture when compared to the vehicle control although some mitoses did occur. Significant inhibition of mitoses by the vehicle (50%) was seen. The vehicle did not inhibit outgrowth.

A dose-response curve (Fig. 13) showed inhibition of mitoses by concentrations of MTX at 1·0 μg or above; there was not a complete absence of mitotic activity. MTX in concentrations which did inhibit mitoses did not inhibit outgrowth. We presumed that thymidine, which bypasses the block induced by MTX, was present in the serum in sufficient quantities to prevent complete mitotic inhibition.

To decrease the influence of serum thymidine, cultures were transferred from 10% FCS to 1% FCS after the first 3 days and followed for 3 additional days with and without MTX (Fig. 14). There was still significant mitotic activity in the MTX treatment cultures. Efforts to time the onset of the block suggested that 6 and 24 h were inhibited. However, other experiments only showed partial blocks at 4, 8 and 16 h.

To eliminate the soluble thymidine in FCS, the FCS was dialysed against phosphate-buffered saline. Dialysis (Fig. 15) did not effect outgrowth size. Cell proliferation was inhibited by dialysis (0 vs 2·5 for control). When cultures started in dialysed FCS were changed to media which had also been dialysed, mitoses returned to about two-thirds the mitotic rate of a similar group of cultures grown for 4 days in normal FCS and them transferred to dialysed FCS. Both culture conditions showed profound synchronization.

Since dialysed FCS was compatable with normal outgrowth, cultures were started in dialysed

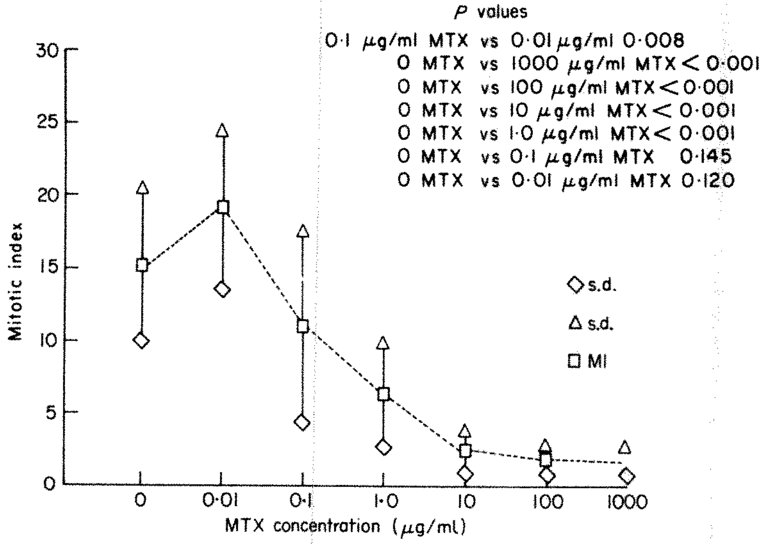


FIGURE 13. MTX dose-response curve. Cultures were started in 10% FCS and after 72 h transferred to 10% FCS containing various concentrations of MTX. There were ten cultures in each group. After 24 h the MI was determined and is indicated with the s.d. and *P* values.

FCS and transferred at 72 or 96 h to normal or dialysed FCS. Again we found normal outgrowth with a low mitotic count (Fig. 16). When switched to normal FCS, a peak of mitotic activity occurred 18 h after transfer and again at 76 h after transfer (58-h cycle). In the cultures switched to dialysed FCS, similar but reduced mitotic activity was seen. Cultures transferred at 96 h (Fig. 17) showed a similar phenomenon. Normal FCS induced a peak of mitotic activity which

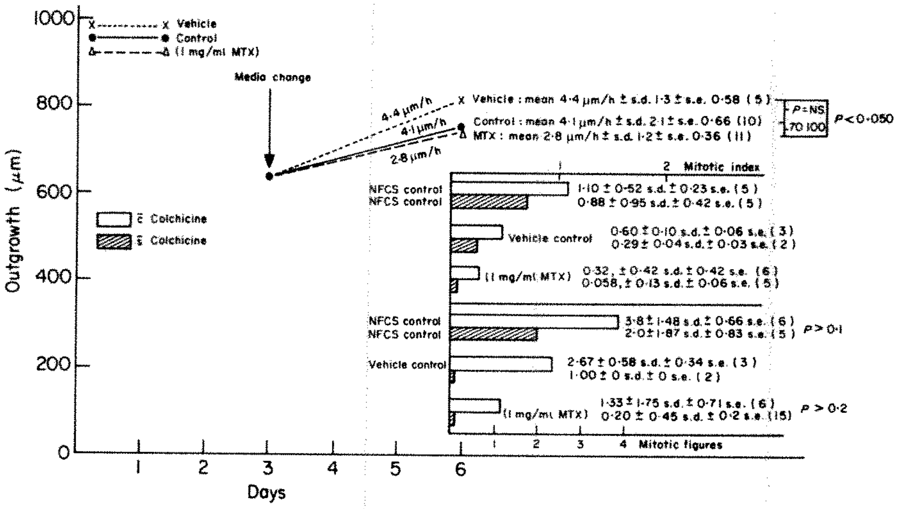


FIGURE 14. Effect of MTX in 1% FCS. This experiment is similar to the one in Fig. 11 except cultures were transferred from 10% normal FCS to 1% FCS containing MTX (1 mg/ml), vehicle, or control with or without colchicine.

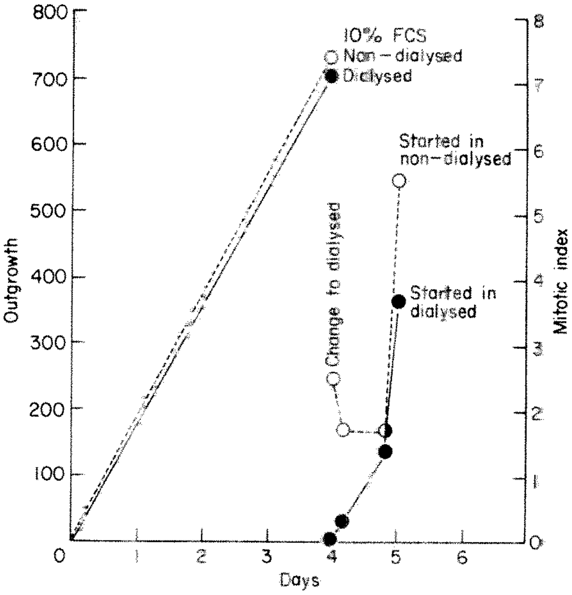


FIGURE 15. Effect of FCS dialysed on outgrowth and MI. Equal numbers of cultures were started in 10% FCS or 10% FCS dialysed for 24 h against phosphate-buffered saline. After 96 h both groups were transferred to 10% dialysed FCS.

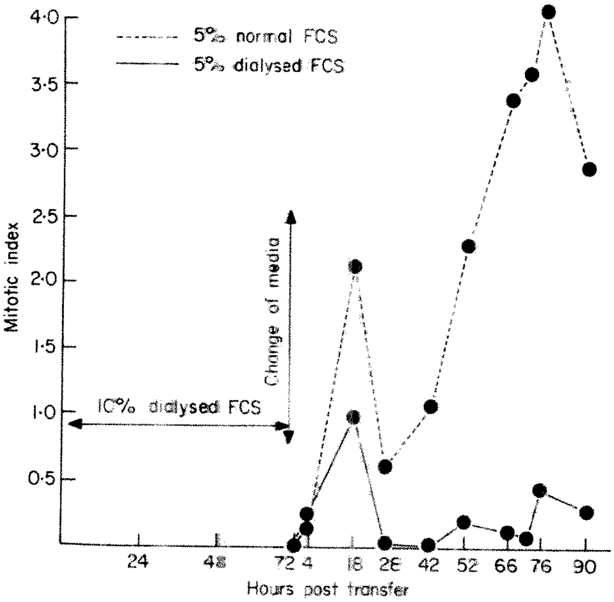


FIGURE 16. Effect of media change at 72 h on mitotic activity in explant cultures. Cultures were grown in 10% dialysed FCS for 72 h; equal numbers were then transferred to either 5% normal or 5% dialysed FCS and MI determined at the times indicated.

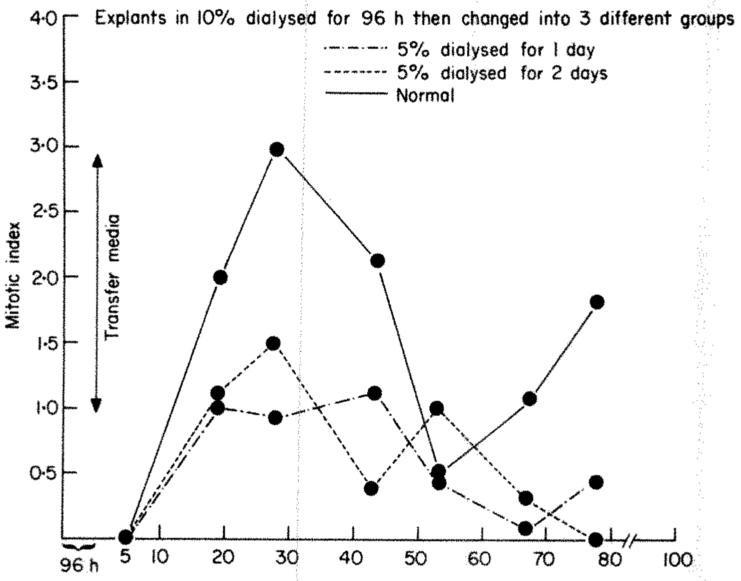


FIGURE 17. Effect of media change at 96 h on mitotic activity in explant cultures. Cultures were grown in 10% dialysed FCS for 96 h; equal numbers were then transferred to normal FCS, FCS dialysed for 1 day and FCS dialysed for 2 days. MI was determined at the times indicated.

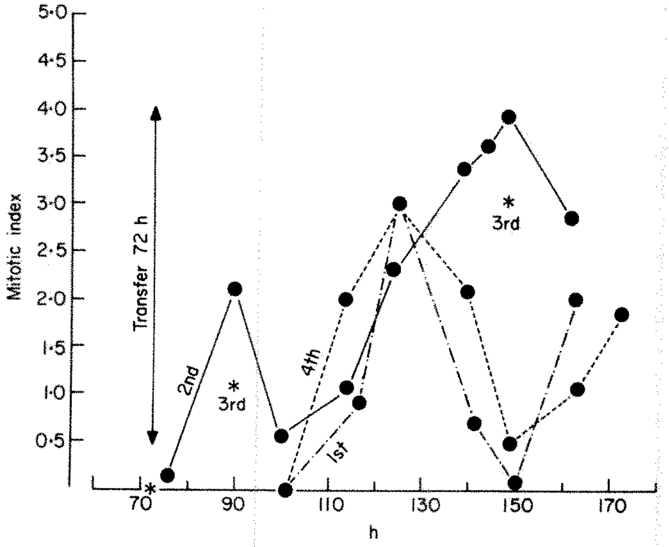


FIGURE 18. Comparison of mitotic activity in cultures transferred at 72 h and 96 h. This figure is a compilation of four separate experiments. Experiments 2 and 3 were transferred from 10% dialysed FCS to 5% normal FCS at 72 h and experiments 1 and 4 were transferred from 10% dialysed FCS to 5% normal FCS at 96 h.

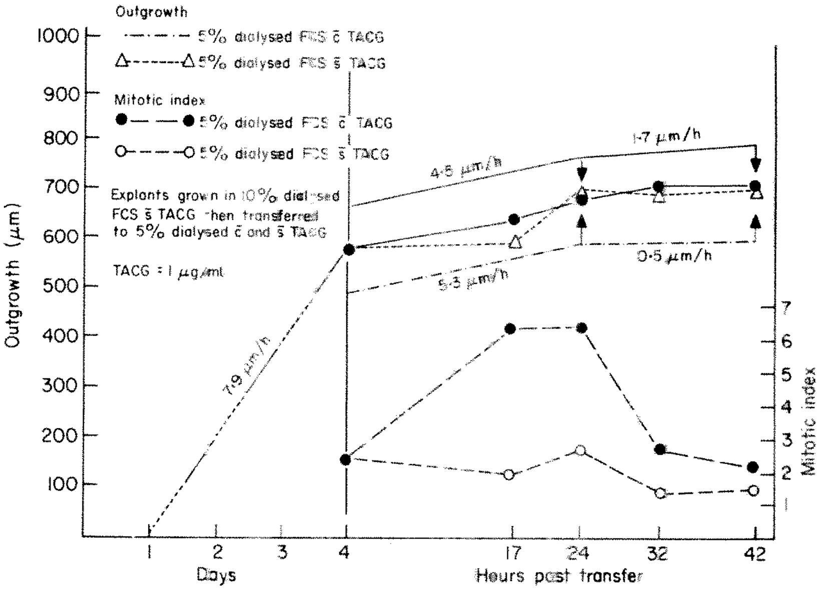


FIGURE 19. Restoration of mitotic activity to cultures in dialysed FCS by nucleosides. Cultures were grown for 4 days in 10% dialysed FCS; equal numbers were then transferred to either dialysed FCS or dialysed FCS containing 1 μg/ml of thymidine, adenosine, cytosine and guanosine (TACG). The actual outgrowth in μm is indicated as well as the outgrowth rate in μm/h. The effect on MI is also indicated.

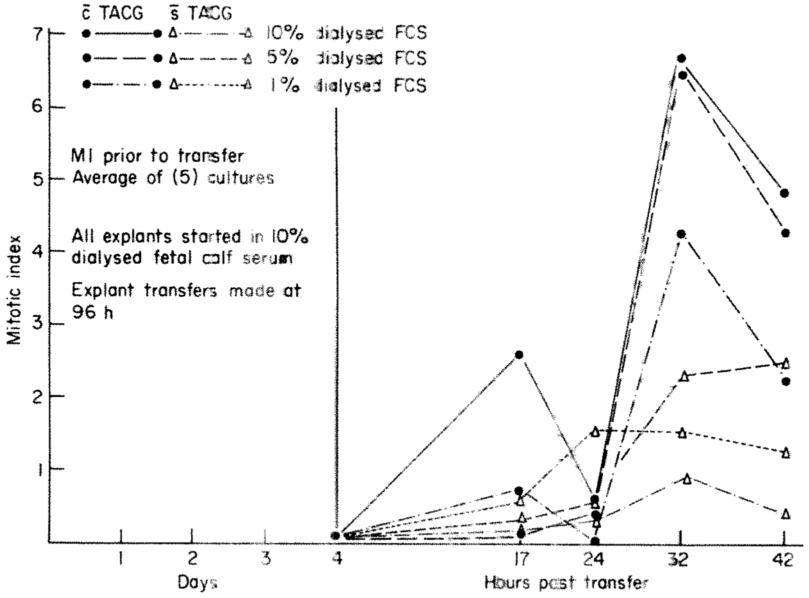


FIGURE 20. Effect of nucleosides on mitotic activity in different FCS concentrations. Cultures were grown for 4 days in 10% dialysed FCS; equal numbers were then transferred to 10% dialysed FCS, 5% dialysed FCS, or 1% dialysed FCS, with or without nucleosides (1 μg/ml TACG). The effect on MI over the subsequent 42 h is indicated.

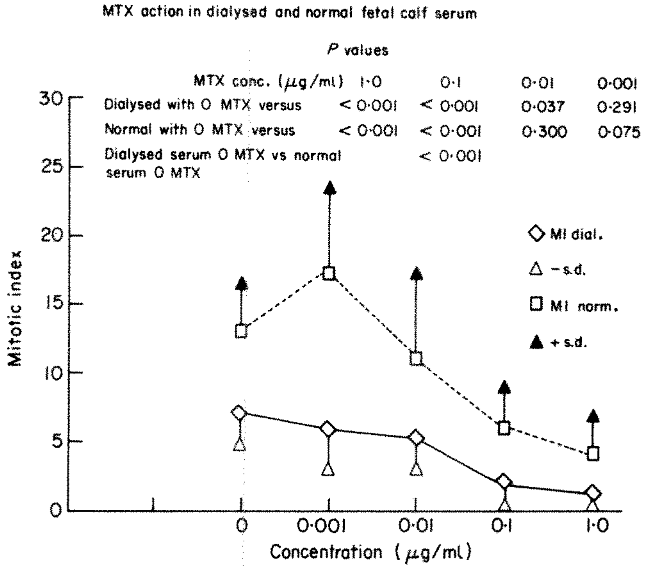
was about twice that seen in cultures continued in dialysed FCS. The peak occurred at 29 h with a second peak 2–3 days later. A high degree of synchrony existed. FCS dialysed for 24 h was the same as FCS dialysed for longer periods.

A compilation of four experiments using normal FCS values in an attempt to time the cycle is shown in Fig. 18. Reproducible synchronized cultures were induced when dialysed cultures were exposed to normal FCS. The cell cycles were approximately 60 h long. The first peak seemed to occur earlier in the cultures transferred after 72 h of exposure to dialysed FCS than in cultures exposed for 96 h. Presumably cells in the dialysed FCS accumulated in the cell cycle (G_1?) and a synchronous cohort of cells started to proliferate when supplied with the required nutrient available in the normal FCS.

Normal rates of proliferation can be induced by addition of a mixture of nucleosides to dialysed FCS (Fig. 19). These cultures have normal outgrowth rates both during the first 96 h of growth (7·9 μm/h) and subsequently (5·3 μm/h *vs.* 4·5 μm/h). The low mitotic index was dramatically elevated by the addition of a mixture of thymidine, adenosine, cytosine and guanosine (TACG), each at 1 μg/ml final concentration. The mitotic index of 6 achieved 24 h later is comparable to the mitotic index after the addition of normal undialysed FCS.

A similar experiment is shown in Fig. 20. Again, outgrowth size was not affected and is not shown; only mitotic activity is indicated. Three different dialysed FCS concentrations are shown along with the presence or absence of TACG. For every FCS concentration the mitotic response was much higher in the presence of TACG. Five and ten per cent dialysed FCS were comparable while 1% was somewhat deficient in some factor other than the nucleosides.

Using the above information, MTX in various concentrations was added to 5% normal or dialysed FCS at the time of transfer and the mitotic index and extent of outgrowth determined after 29 h of incubation. The effects of MTX in concentrations from 0·001 μg/ml to 1 μg/ml of



P values

MTX conc. (μg/ml)	1·0	0·1	0·01	0·001
Dialysed with 0 MTX versus	< 0·001	< 0·001	0·037	0·291
Normal with 0 MTX versus	< 0·001	< 0·001	0·300	0·075
Dialysed serum 0 MTX vs normal serum 0 MTX		< 0·001		

FIGURE 21. Comparison of MTX dose-response in normal and dialysed serum. Cultures were grown for 96 h in dialysed FCS; equal numbers were then transferred to either 5% normal FCS or 5% dialysed FCS containing various concentrations of MTX. The MI was determined at 29 h. The s.d. and *P* values are indicated.

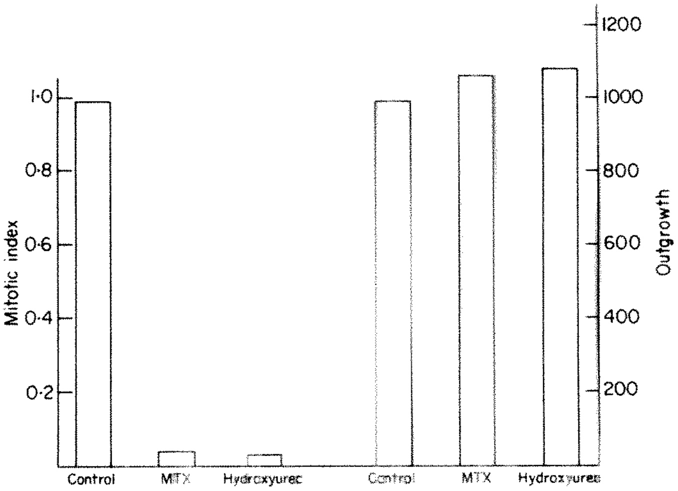


FIGURE 22. Effect of MTX and hyroxyurea on explant cultures. Cultures were grown for 96 h in 10% dialysed FCS; equal numbers were transferred to 5% dialysed FCS with either 10 μg/ml MTX or 10^{-3} M hydroxyurea. MI and outgrowth at 29 h is indicated.

media in both normal and dialysed FCS are illustrated in Fig. 21. The mitotic index was significantly inhibited at 1 μg/ml and at 0·1 μg/ml in both normal and dialysed FCS. When dialysed FCS was transferred to dialysed FCS, significant inhibition occurred at 0·01 μg/ml MTX. Figure 22 illustrates that MTX at a concentration of 10 μg/ml significantly inhibited mitoses in the explant but had no effect on the epithelial outgrowth.

DISCUSSION

We have demonstrated that hydroxyurea and MTX produce similar effects in the explant culture system. High concentrations of each drug inhibited mitotic activity but did not inhibit outgrowth since they did not effect cell migration. Therefore the seemingly contradictory reports of Karasek and Flaxman are both correct. Concentrations of MTX as low as 0·01 μg/ml inhibit the mitotic index in the presence of dialysed FCS, where the effects of thymidine can be controlled.

These findings have several important implications. The explant culture system can be used to study the inhibitory effects of drugs on epidermal proliferation but it is necessary to measure the mitotic index since some of these substances apparently have little effect on the migration of the epithelial cells from the original tissue. We have also demonstrated that human skin need not be used since we have been able to substantiate Flaxman's work using adult pig skin in a comparable system.

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Effects of short-wave ultraviolet radiation (UV-C) on delayed hypersensitivity in the guinea-pig

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SUMMARY

Guinea-pigs, previously sensitized to dinitrochlorobenzene (DNCB), were exposed to varying doses of UV-C radiation (254 nm) on one of the flanks for a period of 15 days. They were then patch-tested with DNCB immediately after UV treatment. The responses to DNCB were diminished both in the irradiated and non-irradiated skin compared with control animals which had not received UV-C radiation. This effect was dose dependent and could only be demonstrated for a limited period of time. Guinea-pigs exposed to UV-C radiation did not show a decreased response to sodium lauryl suphate, which is a potent irritant. The influence of UV-C on delayed hypersensitivity therefore seems to be an immunological effect.

Several reports have been published on the influence of ultraviolet (UV) radiation on the immune system. Morison *et al.* (1981) showed that ultraviolet light of 280–320 nm (UV-B) diminishes the allergic contact dermatitis reaction when guinea-pigs are exposed to UV-B during the sensitization period. It has also been shown that UV-B radiation diminishes the contact allergic reaction to DNCB in previously sensitized guinea-pigs (Austad & Mørk, 1982). Photochemotherapy using psoralen and longwave ultraviolet light (PUVA) inhibits induction of contact sensitivity in guinea-pigs (Morison *et al.*, 1981), and it was also demonstrated that both PUVA and UV-B produce a systemic suppression of the delayed hypersensitivity reaction. In a previous study, animals sensitized to dinitrochlorobenzene (DNCB) showed a decreased response to the allergen when it was applied after PUVA treatment. This effect is temporary and can only be demonstrated in the PUVA treated area (Austad & Mørk, 1981).

Less attention has been focused on the effect of ultraviolet light with wavelength shorter than 280 nm (UV-C) on delayed hypersensitivity. The aim of the present study was to investigate the effect of UV-C radiation on delayed hypersensitivity in the guinea-pig.

MATERIALS AND METHODS

Sensitization

Female albino guinea-pigs of the Hartley strain weighing 350–400 g were used. Contact sensitivity to dinitrochlorobenzene (DNCB) was induced by a combination of intradermal and

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epidermal sensitization. This method gives a very high rate of sensitization. An injection of 0·1 ml of 0·01% DNCB in propyleneglycol was made intradermally. One week later, closed patch topical induction was performed. A 2 × 2-cm filter paper saturated with 0·15 ml 0·1% DNCB in 70% ethanol was applied to the clipped and shaved dorsal thorax. This was covered by overlapping impermeable plastic tape and held in place for 48 h by elastic adhesive bandage encircling the trunk. Challenge was performed 10 days later by closed patch with 0·05 ml 0·1% DNCB in 70% ethanol. This was applied to the dorsal thorax in a Finn chamber (Epitest Ltd, Finland) and fixed with porous adhesive tape and elastic bandage. The patch was removed after 24 h, and the reactions were evaluated the following day. The reactions were graded: 0 = negative; 1 + = slight erythema; 2 + = strong erythema; 3 + = intense erythema and swelling. All animals responded with either 2 + or 3 + reactions.

UV-C radiation

The light source was two Philips TUV 30 W low pressure mercury light bulbs with more than 90% of the UV emission around 254 nm. The irradiance was 1·25 mW/cm^2 at a distance of 15 cm, measured by 65 A radiometer (Yellow Springs Instrument Company).

UV-C induced erythema occurred 3–5 h after irradiation with a maximal reaction after 8 h. The minimal erythema dose (MED) was 25 mJ/cm^2. The erythemal response normally disappeared in 24 h. Repeated exposures to 4 J/cm^2 or higher doses induced a more persisting erythema and hyperkeratosis.

To keep the animals in position during light exposure, they were given a combination of 3 mg fluanisone and 0·1 mg fentanyl citrate (Hypnorm®, Mekos) subcutaneously.

Experimental design

Twenty-five animals previously sensitized to DNCB were divided into five groups from A to E. An area of 6 × 6 cm on the right flank was clipped and shaved and then exposed to UV-C radiation on alternate days for 15 days. At each exposure, group A were given 0·75 J/cm^2, group B 1·5 J/cm^2, group C 2·25 J/cm^2, group D 3·0 J/cm^2, and group E 4·5 J/cm^2. A control group of five animals was not irradiated.

Immediately after the last treatment all animals were exposed to an elicitation dose of DNCB by a Finn Chamber closed patch technique, as described above. This was performed both on the treated right flank and the untreated left flank. The responses were evaluated 24 h after removal of the patches. The animals in group C, D and E were re-challenged with DNCB 12 days later following exactly the same procedure.

To study the effect of UV-C radiation on irritant contact dermatitis, six guinea-pigs were exposed to 3·0 J/cm^2 of UV-C on the clipped and shaved right flank on alternate days for 15 days. They were then treated with 15% sodium lauryl sulphate on an area of 3 × 3 cm on both flanks. A group of six non-irradiated animals was treated with sodium lauryl sulphate in the same way on one flank. The irritant responses were evaluated after 24 h.

Statistical analysis

The statistical significance of the results obtained was calculated by using Student's t-test.

RESULTS

The results are summarized in Tables 1, 2 and 3. The animals in groups A and B, receiving 0·75 J/cm^2 and 1·5 J/cm^2, did not show any significant difference in the response to an elicitation dose

TABLE 1. Allergic response to an elicitation dose of DNCB immediately after a 15-day period of UV-C radiation

Animal no.	Group A (0·75 J/cm²)		Group B (1·5 J/cm²)		Group C (2·25 J/cm²)		Group D (3·0 J/cm²)		Group E (4·5 J/cm²)	
	U	I	U	I	U	I	U	I	U	I
1	+ +	+ +	+ +	+ +	+	+	+	+ +	+ +	+ +
2	+ +	+ +	+ + +	+ + +	+	+	+ +	+	+	+
3	+ + +	+ + +	+ +	+ +	+	+	+	+	+ +	+
4	+ + +	+ + +	+ +	+ +	+ +	+ +	+	−	+ +	+ +
5	+ +	+ +	+ +	+	+ +	+ +	−	+	+ +	+ +
Mean score	2·4	2·4	2·2	2·0	1·4	1·4	1·0	1·0	1·8	1·6

U = Unexposed skin; I = irradiated skin.

of DNCB either on exposed or unexposed skin. In groups C, D and E all animals showed a significant inhibition of allergic response both on UV-C irradiated skin and unexposed skin ($P < 0{\cdot}05$). The decrease in the response to DNCB was more pronounced in group D, receiving 3·0 J/cm², compared with group C, receiving 2·25 J/cm² (Table 1). However, the animals in group E, receiving 4·5 J/cm², showed less reduction of the allergic response than the groups C and D animals. In groups C, D and E the decreased responses were equal in irradiated and non-irradiated areas. Twelve days later there were no significant differences in the response to DNCB in groups C and E compared with the animals in the control group ($P > 0{\cdot}05$). In group D there were still significantly reduced responses in irradiated skin ($P < 0{\cdot}05$) although they were relatively increased compared with the response immediately after the irradiation period.

The animals in the control group unexposed to UV-C radiation, showed the same strong responses both at the first challenge and 12 days later (Table 3).

All twelve guinea-pigs in the two groups treated with sodium lauryl sulphate responded with an erythematous reaction on the exposed skin. There was equal response both on UV-C-irradiated and non-irradiated skin compared with animals which were not irradiated.

TABLE 2. Allergic response to an elicitation dose of DNCB when rechallenged 12 days after UV-C radiation

Animal no.	Group C (2·25 J/cm²)		Group D (3·0 J/cm²)		Group E (4·5 J/cm²)	
	U	I	U	I	U	I
1	+ +	+ +	+ +	+ +	+ + +	+ + +
2	+ +	+ +	+ + +	+ +	+ + +	+ +
3	+ +	+ +	+ +	+ +	+ + +	+ + +
4	+ + +	+ +	+ +	+	+	+
5	+ +	+ + +	+	+ +	+ +	+ +
Mean score	2·2	2·2	2·0	1·8	2·4	2·2

U = Unexposed skin; I = irradiated skin.

TABLE 3. Allergic response to an elicitation dose of DNCB. Re-challenge was performed 12 days later

Animal no.	Control animals unexposed to UV-C radiation	
	First challenge	12 days later
1	+ +	+ +
2	+ + +	+ + +
3	+ +	+ +
4	+ + +	+ + +
5	+ + +	+ + +
Mean score	2·6	2·6

DISCUSSION

UV-C radiation produces a dose-dependent suppression of the contact allergic reaction to DNCB in the guinea-pig. Repeated exposures to 0·75 J/cm^2 and 1·5 J/cm^2 did not give any significant inhibition of the cell mediated allergic response, while exposure to 2·25 J/cm^2, 3·0 J/cm^2 and 4·5 J/cm^2 induced inhibition of the allergic response both in irradiated and non-irradiated skin.

The practical use of UV-C irradiation has mainly been associated with the germicidal properties of these rays. UV-C contains more energy than longwave ultraviolet light, and most of the energy in the UV-C wave band is absorbed in the epidermis (Rosario *et al.*, 1979). Only about 1% of the energy reaches the dermis. Change in the thickness and structure of the epidermis is important with regard to the quantity of UV-C reaching the basal layer of the epidermis. Probably this explains the results in group E. The guinea-pigs in this group developed hyperkeratosis. Even though these animals received more UV-C radiation compared with group C and D, the inhibition of the contact allergic response was less.

UV-C radiation induces formation of pyrimidine dimers. The results may be cell death, mutation or transformation. This has been extensively studied *in vitro* in bacteria (Youngs & Smith, 1976) and in mammalian cells (Elkind & Han, 1978). D'Ambrosio *et al.* (1981) showed the same damage in human skin *in vivo*. In general, immunocompetent cells are more sensitive to UV light than keratinocytes or fibroblasts.

In the contact allergic reaction Langerhans cells play a central role in the afferent phase of the immune response by presenting antigen to immunocompetent lymphocytes. Both PUVA, UV-A and UV-B alter and damage surface markers of these cells (Okamoto & Horio, 1981; Aberer *et al.*, 1981). It is likely that UV-C will affect the Langerhans cells in the same way and thereby impair the antigen response. Bohnert *et al.* (1977) have demonstrated *in vitro* that T-lymphocyte activity and the number of T-cells decrease in proportion to the intensity of UV-C exposure. However, with the low dose of UV-C reaching the dermis it is unlikely that the effect on the T-lymphocytes plays a major role in the inhibition of the allergic reaction.

UV-C radiation did not diminish the irritant contact dermatitis produced by sodium lauryl sulphate. This indicates that the effect observed on allergic contact dermatitis is mainly immunological.

The UV-C-induced cell damage in the irradiated skin can hardly explain the systemic reduction in the delayed hypersensitivity response. Probably, the radiation exerts an indirect influence on the immune system, for instance by releasing humoral mediators. Greaves *et al.* (1977) showed that UV-C activates the production of arachidonic acid and prostaglandins, which may be important in regulating the immune responses.

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Class-specific antibodies in young and aged humans against organisms producing superficial fungal infections

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SUMMARY

Class-specific antibodies (IgG, IgA and IgM) against *Candida albicans*, *Pityrosporum orbiculare* and *Trichophyton rubrum* were measured in the serum from twenty-one young subjects (aged 23–44) and twenty elderly subjects (aged 70–88) who did not have a history of significant superficial fungal infections. We found that (a) antibody to all three organisms was present in all subjects in both groups, (b) except for a reduced level of IgM antibody against *P. orbiculare*, the elderly subjects demonstrated humoral responses in these assays similar to those of the young subjects, (c) IgA antibody was present in higher amounts against *C. albicans* than against *P. orbiculare* or *T. rubrum* in both groups, and (d) the proportion of IgM antibody against *T. rubrum* was higher than that against the other two organisms. These findings suggest that the ecology of these three organisms with respect to the normal human host may be reflected in the serological responses against them.

Superficial infections of the skin or mucous membranes are produced by several fungal organisms such as *Candida albicans*, *Pityrosporum orbiculare* (presumably the same organism as *Malassezia furfur*, the agent of tinea versicolor) and the dermatophytes, including the three most common genera, *Trichophyton*, *Epidermophyton* and *Microsporum*. The ecology and patterns of infections produced by the three major groups of these organisms are quite different: (a) *C. albicans* has its normal place of residence in the human GI tract instead of the skin (Cohen *et al.*, 1969) and can produce mucous membrane lesions whereas the others do not; (b) *C. albicans* and *P. orbiculare* appear to be members of the normal flora in most humans (Cohen *et al.*, 1969; Roberts, 1969), whereas the dermatophytes probably are not, although minor episodes of dermatophytosis are very frequent in the human population.

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In spite of their superficial location, infections with these organisms appear to produce systemic sensitization to the antigens of the infecting fungus (Axelsen, Kirkpatrick & Buckley, 1974; Sohnle & Collins-Lech, 1978; Grappel, Blank & Bishop, 1972). In fact, uninfected persons show such sensitivity to *C. albicans* and *P. orbiculare*, presumably due either to previous subclinical infections or absorption of fungal antigens because of the organisms' role as normal flora (Sohnle & Collins-Lech, 1978). There is some evidence that elderly humans are more likely to develop superficial fungal infections with certain of these organisms, although these infections are often asymptomatic (Tindall & Smith, 1963; Peachey & English, 1974; Gilchrest, 1979). The purpose of the present study was to evaluate the humoral responses to *C. albicans*, *P. orbiculare*, and *Trichophyton rubrum* in uninfected subjects in order to determine if differences in these responses exist that might relate to the age of the subject or the ecology of the organism in the normal human host.

MATERIALS AND METHODS

Subjects

These studies were performed on serum samples drawn from twenty-one young (aged 23–44 years) and twenty elderly (aged 70–88 years) subjects. The elderly subjects were residents of either the Methodist Manor Home for the Aging or the Nursing Home Care Unit of the Wood Veterans Administration Medical Center; most of the young subjects were employees of the latter institution. In both groups, approximately two-thirds were male, one-third female. None of the subjects gave a history of significant superficial fungal infections.

Preparation of extracts

The organisms were cultured in a defined medium consisting of AOAC synthetic broth (Difco Laboratories, Detroit, MI), 1% glucose, and in the case of *P. orbiculare* and *C. albicans*, 1% olive oil and 0·1% Tween 80. After an appropriate period of culture, the organisms were harvested by centrifugation and extracts prepared as previously described (Sohnle & Collins-Lech, 1978). Briefly, for *C. albicans* and *P. orbiculare*, the organisms underwent ether extraction followed by grinding in a mortar and pestle. *T. rubrum* was subjected to the latter process alone. Saline extracts of the treated organisms were prepared, dialysed against distilled water, lyophilized, and then reconstituted in saline before use.

Enzyme-linked immunoabsorbent assay (ELISA)

This assay was carried out in polystyrene tubes which were first coated with 1·0 ml of appropriate dilutions of *C. albicans*, *P. orbiculare*, or *T. rubrum* extracts in 0·01 M phosphate-buffered saline (PBS) at 7·0 for 1 h at 37°C. The tubes were then washed three times with PBS containing 0·05% Tween 20 (PBS-Tween 20), treated with 1·0% bovine serum albumin and washed again. Following this step, 0·5 ml of an appropriate dilution of plasma was added to the tubes and incubated at 37°C for 30 min. The tubes were washed three times again and 0·5 ml of a 1:200 dilution of peroxidase-labelled, affinity-purified goat anti-human IgG, IgA, or IgM (Litton Bionetics, Kensington, MD) was added to each tube. After incubation for 30 min at room temperature, the tubes were washed six times and 1·0 ml of the substrate was added (1·0 mg/ml of *o*-phenylenediamine, Eastman Kodak Co., Rochester, NY, in 0·05 M citrate buffer, pH 4·5, and 0·05% H_2O_2). After 40 min of incubation at room temperature, the development of colour was read in a spectrophotometer at 420 nm.

In order to determine specificity of the peroxidase-labelled antisera, tubes were coated with preparations of IgG, IgA, or IgM antibody (Cappell Laboratories, Cochranville, PA) and then reacted with a 1:200 dilution of each of the three labelled antisera. Good agreement was obtained between the specificity of the antisera obtained in this system and the stated specificity.

Calculations

The data were generally expressed as the optical density (OD) obtained at a constant dilution of plasma (IgG = 1:50; IgA = 1:5; IgM = 1:20) after subtracting the OD in control tubes (without serum). However, in some experiments, $\log_{10}$ dilutions of test plasma were carried out and the data determined over a range of 1:10 to 1:10,000. Proportions of a specific class of antibody were obtained by expressing the OD obtained at the usual dilution for the class over the sum of ODs for all three classes at their usual dilutions as stated above. Statistical comparisons were made using the two-tailed, unpaired Student's t-test with $P < 0{\cdot}05$ being taken as significant.

RESULTS

In Figs 1, 2 and 3 can be seen the results of determinations for the three antibody classes against the three organisms over the range of 10-fold dilutions from 1:10 to 1:10,000 in twelve normal (young) subjects. In all cases there was a fairly linear decline in OD obtained with increasing dilution. As shown in Fig. 2, IgA values obtained against *C. albicans* were higher than those obtained against the other two organisms over this range.

Class-specific antibodies against the three organisms are compared for young and elderly subjects in Table 1. As can be seen, results were not significantly different between the two groups except for lower levels of IgM against *P. orbiculare* in the elderly group. It can be noted

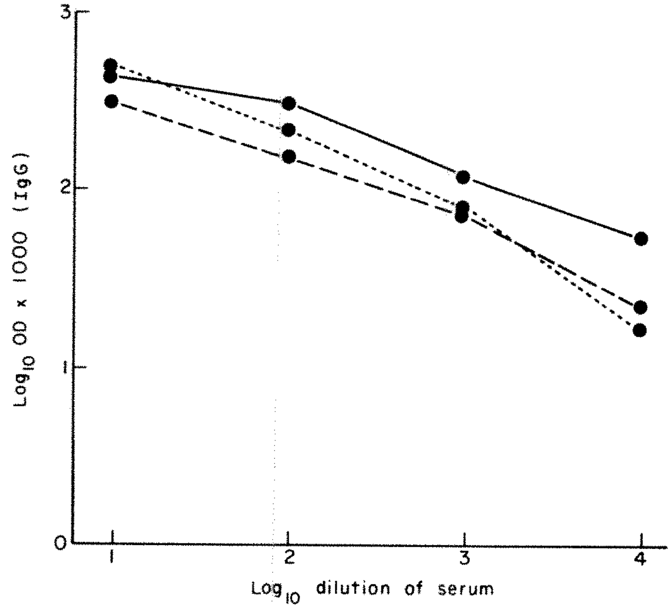

FIGURE 1. Log_{10} optical density (× 1000) obtained for IgG against the three organisms (——*C. albicans*; – – –*P. orbiculare*; - - - - -*T. rubrum*) on the ordinate versus $\log_{10}$ of the serum dilution on the abscissa.

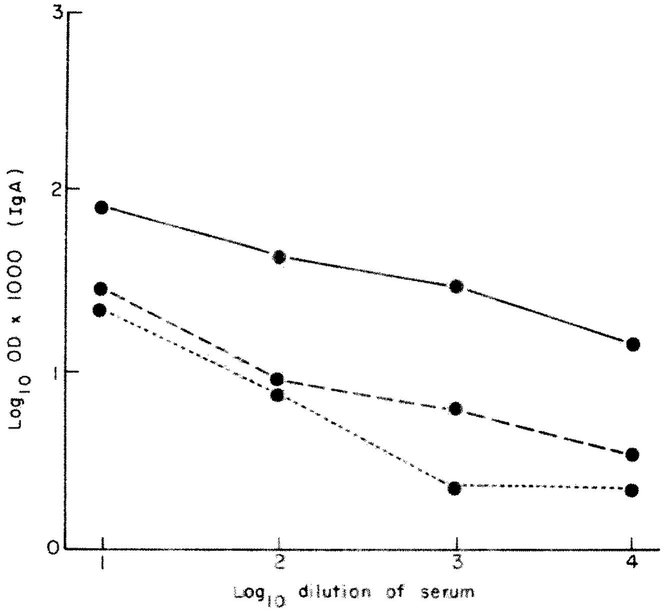

FIGURE 2. Log_{10} optical density (× 1000) obtained for IgA against the three organisms (——*C. albicans*; – – –*P. orbiculare*; - - - -*T. rubrum*) on the ordinate versus $\log_{10}$ of the serum dilution on the abscissa.

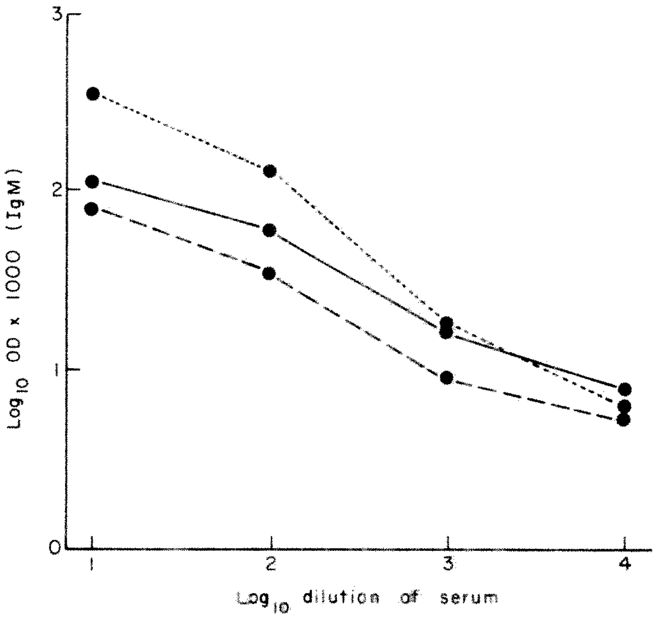

FIGURE 3. Log_{10} optical density (× 1000) obtained for IgM against the three organisms (——*C. albicans*; – – –*P. orbiculare*; - - - -*T. rubrum*) on the ordinate versus $\log_{10}$ of the serum dilution on the abscissa.

TABLE 1. Class-specific antibodies against *C. albicans*, *P. orbiculare* and *T. rubrum* in elderly and young subjects

	Elderly	Young	Statistical significance†
*C. albicans**			
IgG (1:50)	0·64 ± 0·08	0·76 ± 0·06	NS
IgA (1:5)	0·41 ± 0·05	0·44 ± 0·04	NS
IgM (1:20)	0·34 ± 0·09	0·31 ± 0·03	NS
*P. orbiculare**			
IgG (1:50)	0·40 ± 0·07	0·51 ± 0·06	NS
IgA (1:5)	0·17 ± 0·03	0·19 ± 0·03	NS
IgM (1:20)	0·19 ± 0·03	0·40 ± 0·04	$P < 0·01$
*T. rubrum**			
IgG (1:50)	0·07 ± 0·01	0·05 ± 0·01	NS
IgA (1:5)	0·05 ± 0·01	0·04 ± 0·01	NS
IgM (1:20)	0·22 ± 0·06	0·22 ± 0·02	NS

* Data expressed as mean optical density ± s.e. for determinations on sera from twenty-one young and twenty elderly subjects.

† Statistical significance by unpaired, two-tailed t-test (significance taken at $P < 0·05$).

Note that for IgA values in young subjects, those for *C. albicans* were significantly higher than those for *P. orbiculare* ($P < 0·01$) or *T. rubrum* ($P < 0·001$).

TABLE 2. Proportions of IgA and IgM antibody to *C. albicans*, *P. orbiculare* and *T. rubrum*

	IgA*	IgM*
	IgG + IgA + IgM	IgG + IgA + IgM
1. *C. albicans*	0·29 ± 0·02	0·21 ± 0·02
2. *P. orbiculare*	0·16 ± 0·03	0·38 ± 0·03
3. *T. rubrum*	0·12 ± 0·02	0·71 ± 0·04
Statistical significance†	1 vs 2, $P < 0·001$ 1 vs 3, $P < 0·001$	3 vs 1, $P < 0·001$ 3 vs 2, $P < 0·001$

* Data from determinations in twenty-one young subjects expressed as mean ± s.e. of the ratios of OD for IgA or IgM divided by sum of ODs for IgG, IgA and IgM at the standard dilutions (IgG = 1:50; IgA = 1:5; IgM = 1:20).

† Statistical significance by two-tailed, unpaired t-test; significance taken at $P < 0·05$.

that IgA levels against *C. albicans* were higher than against the other two organisms in the elderly group as well as the young group.

Proportions of IgA and IgM antibody to the three organisms are given in Table 2 using the method described above. As expected, the proportion of IgA against *C. albicans* was larger than that against the other two organisms. Also, the proportion of IgM against *T. rubrum* was significantly higher than that against the other two organisms.

Since the proportions shown in Table 2 are based on different serum dilutions for each of the antibody classes, they do not reflect absolute amounts of each class present, but rather relative proportions for comparisons between the three organisms. From the data given in Figs 1, 2 and 3, one can see that the largest amounts of antibody to all three organisms in these normal subjects were of the IgG class, although there was a significant proportion of IgM antibody to *T. rubrum* present also.

DISCUSSION

The data from the present investigation demonstrate that normal subjects, both elderly and young, have detectable antibody against three of the organisms which produce superficial fungal infections, *C. albicans*, *P. orbiculare* and *T. rubrum*. There did not seem to be significant differences between the level of antibody present in elderly and young subjects except for a decrease in IgM antibody against *P. orbiculare* in the former group. However, there were significant differences between the proportions of class-specific antibody present in the serum of young subjects against the different organisms. Most striking was the increased amount of IgA antibody against *C. albicans*. This finding was apparent in both the elderly and young groups. Also, the proportion of IgM against *T. rubrum* appeared to be significantly higher than that present against the other two organisms.

Previous studies have demonstrated antibody in the serum of normal persons against *C. albicans* (Chew & Theus, 1967; Axelsen, 1976), *P. orbiculare* (DaMert, Kirkpatrick & Sohnle, 1980) and *T. rubrum* (Grappel *et al.*, 1972). In general, the amount of antibody present was lower than that in the serum of patients chronically infected with these organisms. Studies using relatively insensitive techniques such as immunodiffusion have generally yielded negative results using sera from uninfected subjects. Studies of antibody to *Trichophyton schoenleinii* in uninfected subjects demonstrated the absence of precipitating antibody, but the presence of antibody by charcoal agglutination, an assay that measures primarily IgM antibody (Grappel, Blank & Bishop, 1971). Determination of the class of antibody present in the serum of normal subjects against the organisms producing superficial fungal infections has not received much attention in previous studies

The mechanism by which normal subjects have become sensitized to the three organisms studied in this investigation is unclear, but there are several possibilities: (a) the subjects may have undergone previous subclinical infections with each of the organisms; (b) contact with the organisms (particularly as part of the body's normal flora) may have produced sensitivity through absorption of the organisms' antigens without actual infection; (c) there may be cross-reactivity between the antigens of these organisms and other antigens to which normal humans are sensitized. Some dermatophytes have been found to contain substances which precipitate with C-reactive protein (Baldo, Fletcher & Pepys, 1977). The finding of a higher proportion of IgA antibody directed against *C. albicans* than against the other two organisms suggests that sensitization to the former occurred across a mucosal surface, such as the gastro-intestinal tract, in which it resides. In patients with severe, recurrent vaginal candidiasis, elevated anti-*Candida* antibody levels have been found, and a large proportion of this antibody was found in gel-filtration fractions containing IgA and secretory piece. In that study, it was suggested that some of the IgA anti-*Candida* antibody from the local vaginal response finds its way into the systemic circulation (Mathur *et al.*, 1977). Our findings suggest that a similar event may occur in normal subjects, although it is not clear whether or not the IgA anti-*Candida* antibody in these subjects is secretory in nature.

The biological value of antibody in the defence against superficial fungal infections is not clear. Indeed, most subjects having chronic infections with the organisms evaluated in the present study have higher than normal titre of antibody to that organism (DaMert, Kirkpatrick & Sohnle, 1980; Grappel *et al.*, 1972), perhaps indicating that this antibody is not protective. The possibility exists that certain types of antibody responses may actually be detrimental to host defenses; interference with cell-mediated immunity by IgE antibody has been proposed as a pathogenetic mechanism in certain cases of chronic dermatophytosis (Jones, Reinhardt & Rinaldi, 1974).

The relative proportions of IgM antibody to the three organisms may also reflect their manner of contact with the human host. Normal humans probably have less contact with *T. rubrum* than with the other two organisms since *C. albicans* and *P. orbiculare* appear to be part of the normal flora in most persons. This factor may be related to the higher proportion of IgM against *T. rubrum* than against the other two organisms, but the cause of this relationship is not clear. The reason for the decreased level of IgM antibody against *P. orbiculare* in the elderly subjects is also not certain, but it appears to reflect the interaction of this particular organism with aged humans since the levels of IgM antibodies against the other two organisms were similar in this group and in the young group.

ACKNOWLEDGMENTS

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Alkaline phosphatase immuno-enzymatic technique in the diagnosis of pemphigus vulgaris

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SUMMARY

Alkaline phosphatase was used in an immuno-enzymatic procedure to detect tissue-bound and circulating antibodies in pemphigus vulgaris. Pemphigus antibodies were revealed by a direct method using alkaline phosphatase conjugated goat anti-human IgG. Cryostat sections were incubated with the specific antiserum, and alkaline phosphatase activity was then revealed histochemically either by Gomori's technique or by the azo dye method. The sections were examined by light microscopy and intercellular staining was demonstrated in the epidermis. The indirect method, in which the patient's serum was incubated with sections of normal skin, gave similar results. Using parallel sections, an immunofluorescent technique was used to demonstrate tissue-bound and circulating antibodies. The alkaline phosphatase method appeared to be slightly less sensitive than the immunofluorescent method.

Immunofluorescent (IF) and immuno-electron microscopic techniques have demonstrated immunoglobulin deposits in the intercellular spaces of the epidermis in pemphigus (Beutner, Chorzelski & Jordon, 1970; Wolff & Schreiner, 1971). This direct IF testing is a reliable diagnostic procedure showing positive findings early in the disease (Judd & Lever, 1979). In addition, intercellular anti-epithelial antibodies can be detected in the serum of some patients by the indirect IF technique (Beutner *et al.*, 1970; Judd & Lever, 1979), and this is specific for pemphigus (Chorzelski, Von Zeiss & Lever, 1966; Harrist & Mihm, 1979; Peck *et al.*, 1968; Sams & Jordon, 1971).

Some authors found a positive correlation between the antibody titre and the clinical activity of the disease (Chorzelski *et al.*, 1966, 1980; Peck *et al.*, 1968; Sams & Jordon, 1971). Sams & Jordon (1971) even claimed that sequential titres are useful as a predictive guide to therapy. Others found a statistically significant relationship between level of titre and level of disease activity, but not in every case (Fitzpatrick & Newcomer, 1980; Judd & Lever, 1979; Judd &

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Mescon, 1979; Weissman et al., 1978). Thus, the IF method has proved of particular importance for diagnosis, and perhaps also for prognosis and treatment, of pemphigus.

However, a major drawback of the IF technique is the need for special equipment, and the poor morphology and the impermanence of the preparations.

More recently, immuno-enzymatic (IE) methods for detection of antibodies in body fluids were introduced, using chemically linked antibody and enzyme complexes, which retain both immunologic and enzymic activity. These assays are analogous to IF assays except that an enzyme label is used in place of the fluorescein. Enzymes such as alkaline phosphatase and peroxidase are usually employed. The enzyme substrate is added to the incubation mixture and the colour change indicates the reaction (Engvall & Perlman, 1972; Vejtorp, 1978; Voller, Bidwell & Barlett, 1977). Since the substrate of peroxidase was found to be carcinogenic, alkaline phosphatase is now more often used (Vejtorp, 1978; Voller et al., 1977).

IE techniques were later employed on tissue samples, for example Bellon, Saplin and Druet (1975) demonstrated antibodies directed against the basement membrane of the glomeruli by the immunoperoxidase method. Mason and Sammons (1978) applied the alkaline phosphatase IE procedure for the detection of immunoglobulins, lysozyme and lactoferrin in paraffin sections and cell smears.

In dermatology, the immunoperoxidase method was used by Zanin et al. (1978) and by White, Morgan & MacDonald (1979) on skin sections of patients with LE and several bullous diseases. Pehamberger, Konrad & Holubar (1978) used this method to locate immunoglobulins and complement in the junctional zone of skin of patients suffering from SLE and chronic DLE. However, results of the indirect technique have not yet been published.

The stain does not fade in sections treated with enzyme labelled antibodies; they can be counterstained with routine histological stains and can be examined with a conventional light microscope.

The purpose of this work was to demonstrate antiepithelial antibodies in pemphigus vulgaris by direct and indirect immunoenzymatic techniques utilizing alkaline phosphatase as the immunologic marker enzyme and to compare the results with those obtained by the IF method. To the best of our knowledge, this is the first study using alkaline phosphatase to detect antibodies in skin disease, and also the first time an indirect IE technique has been used to demonstrate antibodies in the sera of patients with pemphigus.

MATERIALS AND METHODS

Tissue samples and sera

Biopsy specimens from five patients with active pemphigus vulgaris and five biopsies from normal controls were studied. The biopsy specimens were frozen within 1 h of biopsy and stored at −80°C for a period of 1–40 days until cut at 4 μm. The sections were then stored at −20°C till used, usually within 2 weeks of cutting.

Eight serum samples from patients with active pemphigus and eight normal control sera were stored at −20°C for a period of 1–40 days. Normal human epidermis (frozen and cut at 4 μm) was used as a substrate for the detection of antibodies in the patients' sera.

All the sections were air dried for about 2 h at room temperature prior to storage.

Antisera

Preparation of enzyme conjugate. IgG of goat antihuman IgG, γ chain (Miles Lab.) was purified by chromatography on DEAE cellulose and coupled to alkaline phosphatase type VII from calf intestine (lot no. 83C-0159, Sigma) by using the one-step glutaraldehyde method described by Engvall & Perlman (1972). To 0·7 mg/0·7 ml of protein from the IgG fraction, 0·3 ml alkaline phosphatase (5 mg protein/ml) and 0·03 ml glutaraldehyde 2% were added. After incubation and dialysis the conjugate was kept at 4°C and diluted to 1:100 before use. The dilution 1:100 was found to be optimum in the experimental set-up we worked with.

Fluorescein conjugated goat anti-human IgG antiserum (Hyland Laboratories). This was diluted 1:35 according to the instructions.

Immunoenzymatic labelling with alkaline phosphatase

Indirect test. Twofold dilutions of patients' sera were applied to cryostat sections of normal skin. After incubation at 37°C for 30 min, the slides were washed three times for 5 min each (Step 1).

Tris buffered saline (0·5 M Tris-HCl, pH 7·6, diluted 1:10 M saline) was used as diluent for sera, antisera and for washing slides between incubation steps.

In some experiments 0·1% BSA was added to the diluent in order to reduce non-specific reactions.

Goat anti-human IgG was applied, and the sections incubated for 30 min at 37°C. The slides were then washed as for step 1 and reacted for alkaline phosphatase either by the method of Gomori (Barka & Anderson, 1963; Pearse, 1968), or by the simultaneous coupling azo dye method (Mason & Sammons, 1978; Pearse, 1968) (step 2).

Staining by Gomori's technique was done according to Barka & Anderson (1963) and it required 1 h of incubation, the optimal time for the reaction. Positive reaction for alkaline phosphatase was indicated by a black precipitate at the presumed sites of enzymic activity and therefore indicated the presence of the enzyme and hence of the conjugate (step 3a).

When staining according to the simultaneous coupling azo dye method, a substrate solution was prepared by dissolving 1·0 mg/ml Fast Red TR or Fast Blue RR salt (Sigma) in Tris HCl buffer, 0·1 M pH 9·0, containing 0·1 mg/ml naphthol AS phosphate (N-5625, Sigma), and this was filtered directly onto the slides. After incubation for 5–60 min at room temperature with gentle shaking the slides were washed in tap water and covered with glycero-gel (step 3b).

Controls. In order to confirm the method, pemphigus and control sera were tested in duplicate. Step 2 was omitted in one of the duplicates. Steps 2 and 3 of the indirect test were applied to cryostat sections of pemphigus and normal control skin in duplicate. One of the duplicates was used as a control and step two was omitted.

Immunofluorescent tests

The relevant steps of the immuno-enzymatic method were repeated (i.e. steps 1 and 2 for the indirect test and step 2 for the direct test) but the antiserum was labelled with fluorescein instead of alkaline phosphatase.

RESULTS

In the direct test, when pemphigus sections were stained by Gomori's method, an intercellular black staining indicated the presence of antibodies between the epidermal cells (Fig. 1a). The blood vessels were also stained, due to the inherent presence of alkaline phosphatase. This

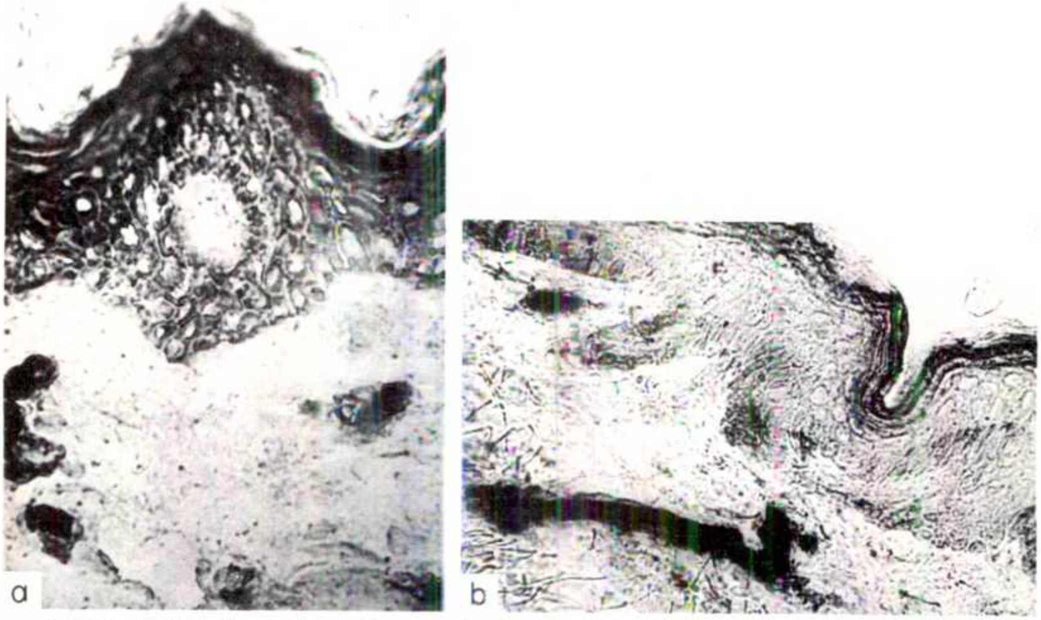

FIGURE 1. (a) Pemphigus vulgaris. Direct alkaline phosphatase immunoenzymatic test. Section stained by Gomori's method. Intercellular black stain indicates the presence of antibodies between keratinocytes. The dermal blood vessels are also stained due to their intrinsic alkaline phosphatase activity. (b) Normal control. Gomori's stain. Only the dermal blood vessels are stained.

staining was used as an intrinsic control for the histochemical method itself, as it was independent of the immunological reaction. The direct test on normal control skin sections was negative in the intercellular area of the epidermal cells (Fig. 1b).

In the indirect test, the same intercellular staining between the keratinocytes was revealed only with the positive pemphigus sera, indicating the presence of circulating anti-epithelial antibodies.

In the direct and indirect tests, the controls without conjugate were negative.

When stained with naphthol AS phosphate and Fast Red TR salt, the staining was red. This red reaction was more intense than the blue one obtained by the Fast Blue RR salt.

Comparing the intensity and the localization of the enzymic activity there was no difference between the results obtained when staining with Gomori's method or with Fast Red TR and naphthol AS phosphate, both in the direct test and in the indirect test in various dilutions.

In the direct test, sections from five pemphigus patients were examined by the IE and by the IF techniques concomitantly. The results were comparable with respect to localization, but the staining appeared weaker with IE than with IF.

In the indirect test, eight sera from five patients suffering from pemphigus vulgaris were examined. One patient had three serum antibody titre determinations on different occasions, and one had two. Twofold dilutions of these sera were prepared, starting from 1:10. The antibody titre in the alkaline phosphatase IE method was compared with the titre in the IF method (Table 1). Three sera had a titre of 1:80 by IF. Two of them had a titre of 1:80 by the IE method and one had 1:160. One serum was 1:160 by IF and 1:80 by IE. Two sera showed titres

TABLE 1. Antibody titres of pemphigus vulgaris sera as determined by the immuno-enzymatic and immunofluorescent methods

	Titre	
Patient	Immuno-enzymatic method	Immunofluorescent method
1	160	640
	80	160
	80	80
2	640	1280
	320	640
3	80	80
4	640	1280
5	160	80

of 1:640 by IF, one of them was 1:320 by IE and the other was 1:160. Two sera were 1:1280 by IF and 1:640 by IE.

DISCUSSION

In the direct method, the intensity of staining with the alkaline phosphatase IE method appeared weaker than the observed IF. It is possible that the antibody we used in the IE method was less sensitive, or that its conjugation to the alkaline phosphatase was weaker. Another possibility is that during incubation and washing of the sections, part of the antigen was dissolved and washed out, as in the IE method there are more steps and washings than in the IF method. Zanin *et al.* (1978), who employed the direct immunoperoxidase method on biopsies of skin lesions including pemphigus, found this method less sensitive than the IF method. For pemphigus it gave positive results only in one out of three cases, all of which were positive by IF. In our experiment the IE method gave positive results when applied to sera which contained antibodies, as determined by the IF method, while the appropriate controls were negative. When comparing the titres, a slightly higher titre was observed in the IF test, but generally the difference was of only one dilution.

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Extensive pityriasis alba: A histological histochemical and ultrastructural study

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SUMMARY

Nine patients with extensive pityriasis alba were studied using histopathological and histochemical techniques and electron microscopy. There was a reduction in the density of functional melanocytes in the affected areas without any change in their cytoplasmic activity. The melanosomes tended to be fewer and smaller, but their distribution pattern in the keratinocytes was normal. Melanosomal transfer to keratinocytes was generally not disturbed. The histology was non-specific. Hyperkeratosis and parakeratosis were not consistently present, and it seems unlikely that they played a significant role in the pathogenesis of the hypomelanosis. A variable degree of intercellular oedema and intracytoplasmic lipid droplets were present. The hypopigmentation may thus be due primarily to the reduced numbers of active melanocytes and a decrease in number and size of melanosomes in the affected skin.

Pityriasis alba is a relatively common skin disorder, usually seen in children. The lesions are frequently limited to the face, though involvement of the arms and shoulders is common. In an uncommon clinical variant, described by Adamson (1908), the lesions are distributed symmetrically over the buttocks, upper thigh, lower back and the extensor aspects of the arms. The widespread involvement is relatively commoner in older children and young adults, and the condition has been conveniently regarded as a variant of pityriasis alba, pending the elucidation of the nature of both conditions (Rook & Wilkinson, 1979). Cases with such widespread lesions are referred to throughout this text as 'extensive pityriasis alba' (EPA).

Few studies of classical pityriasis alba and its less common variant have been reported, and no definite cause has been determined. However, it is generally considered that the two conditions represent a localized inflammatory reaction, unrelated to fungi and bacteria, but possibly related to dryness of the skin (Wells, Whyte & Kierland, 1969).

The hypomelanosis has been attributed to post-inflammatory changes, ultraviolet screening effects of the hyperkeratotic and parakeratotic epidermis, and possibly to a reduced capacity of

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hypermetabolic epidermal cells to take in melanin granules (Rook & Wilkinson, 1979). In one study based on silver nitrate staining of sections in three cases of EPA, the hypopigmentation was related to a reduction in the amount of pigment in the basal layer of the epidermis (Wells *et al.*, 1960). To our knowledge, no detailed histochemical or electron microscopical data have been reported.

The aim of this paper is the characterization of EPA at the cellular and ultrastructural levels.

MATERIAL AND METHODS

Nine patients with EPA were studied. All except one patient were adults (ages 6 to 27 years). Seven were females and two were males. Two patients had skin type III and seven skin type IV (skin types as classified by Morison, Parrish & Fitzpatrick, 1978). The duration of the eruption ranged from 2 weeks to 6 years. There was no seasonal onset of the rash although many of the patients noticed worsening of the condition during summer. There was no preceding inflammatory skin disease. A history of atopy was recorded in only one patient. A positive family history of pityriasis alba was obtained in two patients and atopy in two. The lesions were asymptomatic and were distributed symmetrically mainly over the trunk in two patients, trunk

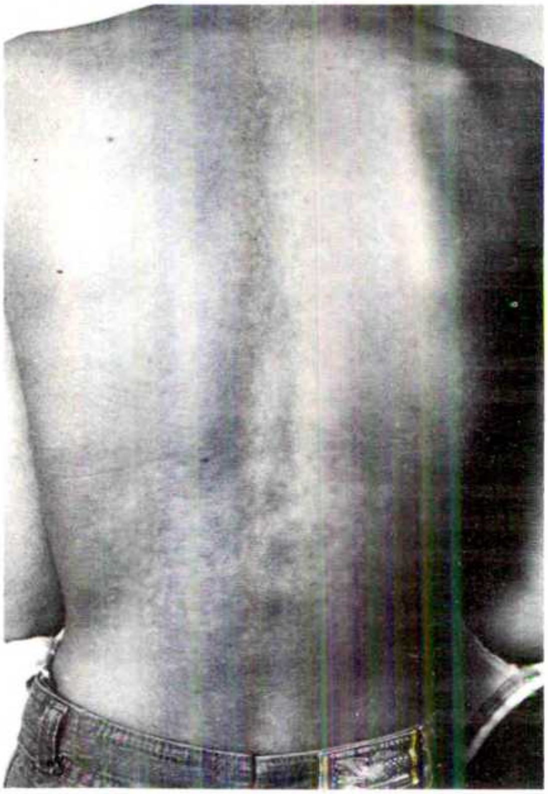

FIGURE 1. Generalized, symmetrical, hypopigmented, non-scaly macules, more numerous and confluent over midback. No fungal elements were detected.

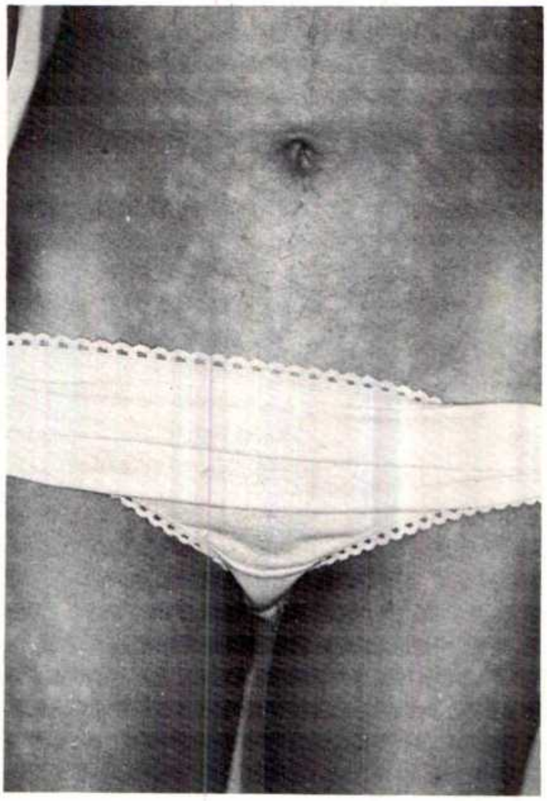

FIGURE 2. Numerous non-scaly, circumscribed, hypopigmented macules over the trunk.

and arms in five patients and more generalized in two patients. These consisted of hypopigmented macules which were occasionally slightly scaly. The surface of the lesions was generally smooth and there was no erythema, atrophy or scarring (Figs 1 and 2).

Scrapings for potassium hydroxide examination were taken from all cases. Biopsies for routine examination and electron microscopy and shave biopsies for split DOPA examination were made under local anaesthesia from affected and adjacent normal-appearing skin.

Light microscopy

The biopsies were fixed in 10% formaldehyde solution and embedded in paraffin. Sections were stained with hematoxylin and eosin, Fontana's silver, PAS and elastic tissue stains. The shave biopsies were incubated in 2 N sodium bromide at 37°C for 2–3 h for epidermal separation. The epidermal sheets were incubated with 0·1% DOPA in a 0·1% sodium phosphate buffer, pH 7·4, for 8 h. The sheets were then fixed with 10% formaldehyde, dehydrated in alcohol and mounted. They were examined with a light microscope at a magnification of × 400 and the melanocytes in ten square fields, each 0·033 mm^2, were examined and counted (eyepiece micrometer calibrated with a stage micrometer). The number of melanocytes per mm^2 was then derived.

Electron microscopy

The methods used were similar to those described previously (Zaynoun, McVittie & Hunter, 1977; Zaynoun *et al.*, 1977b). The biopsy specimens were immediately immersed in half-strength Karnovsky's fixative (Karnovsky, 1965), cut into small pieces and fixed for 5 h at room temperature. They were left overnight in sodium cacodylate buffer, post-fixed in 1% aqueous osmium tetroxide, stained *en bloc* in uranyl acetate, dehydrated and embedded in araldite resin. Ultrathin sections were cut, post-stained with uranyl acetate and lead citrate and examined with an R.C.A. EM 4 electron microscope.

Three blocks from each biopsy specimen were examined. The changes in the keratinocytes and melanocytes were recorded. For the evaluation of melanosomal size and distribution, eighteen micrographs of the basal cell layer were taken randomly from each specimen at a magnification of × 10,000. The prints were enlarged to a final magnification of × 40,000. All well-formed melanosomes within keratinocytes, melanocytes and dendrites were included. The total number and the number occurring both in non-aggregated (single) form and in complexes were calculated for each biopsy specimen. The transverse diameters of all melanosomes were measured using a × 10 eyepiece with a crossline graticule (0·5 mm divisions) and the mean transverse diameter was derived. In addition, the percentage of single melanosomes in the total melanosomal population of each biopsy specimen was calculated. The results were analysed by Student's t-test.

RESULTS

Light microscopy

Histopathology. Biopsies were taken from all patients except one. No fungal elements were detected in smears or biopsies. The findings were as follows:

(1) Epidermis: (a) Mild to moderate hyperkeratosis in the skin lesions in four patients and parakeratosis in one patient. (b) A significant reduction in the amount of melanin in the epidermis in the clinically affected skin in all cases except one where there was a mild degree of hyperpigmentation in the affected skin. (c) Focal exocytosis and spongiosis in the affected skin in two patients.

TABLE 1. DOPA positive melanocytes per mm^2 in normal and affected skin

Case	Normal	Affected
1	1770	1587
2	2163	1944
3	2252	1935
4	1983	1261
5	2919	1662
6	2487	1851
7	1731	1187
8	Not done	Not done
9	1536	1371

The above figures were analysed by the Student's paired t-test and a P value of $< 0{\cdot}005$ was obtained.

(2) Dermis: A mild perivascular inflammatory infiltrate in the upper dermis in both affected and apparently normal skin in four patients. Mild oedema of the papillary dermis in the affected skin in one patient. No abnormalities in the collagen or elastic tissue were detected. The appendages appeared normal. A few melanophages were present in the upper dermis in both affected and normal skin in three patients.

Split-DOPA examination.

(1) Melanocyte population: The number of active melanocytes per mm^2 in each biopsy specimen is shown in Table 1. A decrease in melanocyte density was noted in the affected skin in all patients.

(2) Intensity of DOPA reaction: The staining was lighter in the affected skin of cases 3,5,7 and 9, more intense in the affected skin of case 4 and similar to that of control skin in cases 1, 2 and 6.

(3) Shape and size of melanocytes and dendrites: It was difficult to detect possible differences in the size of melanocytes and number of dendrites between control and affected skin due to the marked variation in different fields in the same specimen. The melanocytes in the affected skin in cases 3 and 4 were generally smaller and rounded and had few short dendrites compared to control skin. The reverse was true for case 6. The dendrites in case 9 were better developed in the affected skin although the melanocyte size was similar to that of control sites. No detectable differences between control and affected areas were observed in cases 1, 2, 5 and 7.

Electron microscopy

General epidermal changes. A significant degree of intercellular oedema was observed in the

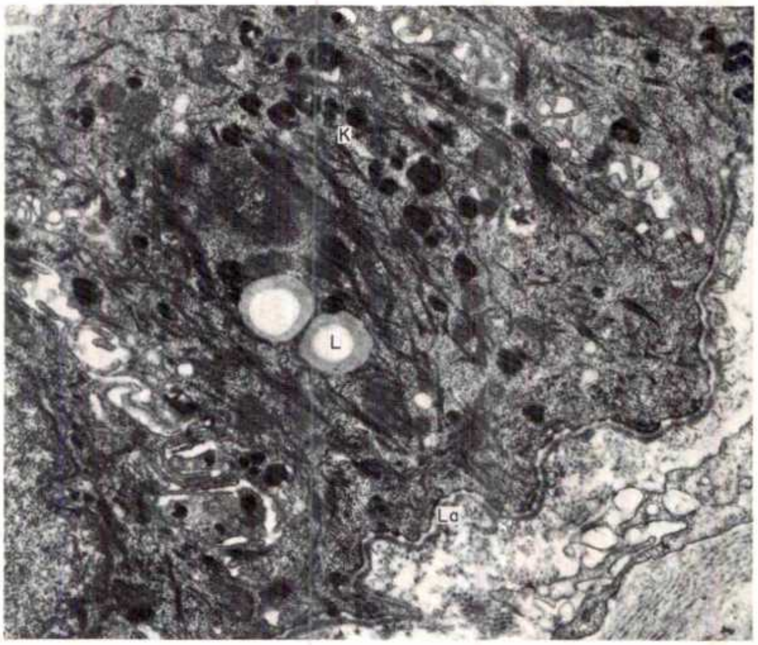

FIGURE 3. Intracytoplasmic lipid droplets (L) in the lesional skin. K = Keratinocyte; La = basal lamina (× 7,000).

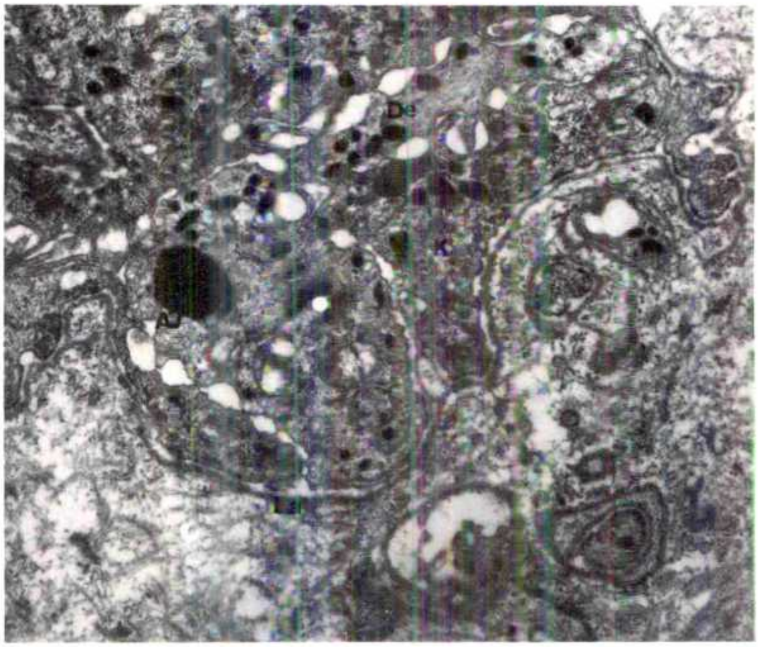

FIGURE 4. Melanosomal autophagic vacuole (AV) inside a melanocyte. K = Keratinocyte; De = dendrite; LA = basal lamina (× 7,000).

affected skin in four patients. No intercellular oedema was observed in one patient. The control skin in four patients showed mild intercellular oedema. Intracytoplasmic lipid droplets were noted in the hypopigmented lesions in seven cases and in the control skin in three cases. These were generally larger and more numerous in the affected areas (Fig. 3). Langerhan's cells were seen in both control and affected areas and appeared normal.

Changes in melanocytes. No serious attempt was made to quantify any differences in the number of melanocytes between affected and control sites because of the well-known drawbacks of the use of electron microscopy for this purpose. The melanocytes in the affected as well as control skin appeared normal. The cytoplasm was active and contained all melanosomal precursors and mature melanosomes. The dendrites were generally laden with mature melanosomes and the adjacent keratinocytes had an adequate number of melanosomes. In the affected skin in one patient only, however, the dendrites in some fields where there was significant intercellular oedema were laden with melanosomes but the adjacent keratinocytes contained very few melanosomes. An occasional melanosomal autophagic vacuole was noted in the melanocyte in the affected skin in another patient (Fig. 4).

Melanosomal changes. There were no apparent differences in the shape and degree of melanization of melanosomes between the affected and normal skin. A total of 18,349 melanosomes were measured and the results are summarized in Table 2. A definite reduction in the total number of melanosomes in the affected skin was obtained in cases 1, 3, 4, 5 and 9. A significant decrease in the transverse diameter of melanosomes ($P < 0.05$) was present in cases 1, 2, 3, 4 and 7. No difference in size between affected and control skin was observed in cases 5, 6 and 9. Case 8, however, showed a significant increase in melanosomal size in the affected areas.

TABLE 2. Melanosomal size and distribution in normal and affected skin

Case Number	Total number of melanosomes	Mean transverse diameter (μm)	s.d.	s.e.mean	Significance	% Single	% Complex
1. Normal	1126	0·189	0·074	0·002	$P < 0{\cdot}05$	62·0	37·9
Affected	749	0·185	0·049	0·002		57·5	42·4
2. Normal	829	0·228	0·123	0·004	$P < 0{\cdot}05$	56·4	43·6
Affected	890	0·217	0·108	0·004		51·7	48·2
3. Normal	1317	0·203	0·055	0·002	$P < 0{\cdot}001$	59·6	40·4
Affected	1035	0·194	0·065	0·002		51·7	48·2
4. Normal	696	0·231	0·211	0·008	$P < 0{\cdot}01$	49·7	50·2
Affected	387	0·204	0·069	0·004		68·6	31·3
5. Normal	1384	0·218	0·128	0·003	NS	82·0	17·9
Affected	1091	0·218	0·128	0·004		83·4	16·6
6. Normal	868	0·207	0·080	0·003	NS	71·2	28·7
Affected	899	0·209	0·002	0·000		73·1	26·8
7. Normal	1220	0·193	0·057	0·002	$P < 0{\cdot}001$	62·8	37·1
Affected	1129	0·185	0·053	0·001		49·6	50·4
8. Normal	1242	0·198	0·058	0·002	$P < 0{\cdot}001$	52·7	47·2
Affected	1255	0·206	0·064	0·002		47·5	52·4
9. Normal	1419	0·215	0·088	0·002	NS	56·5	43·4
Affected	813	0·212	0·058	0·002		60·0	39·9

The melanosomal distribution pattern did not show any significant changes in the affected areas compared to control skin.

DISCUSSION

The main microscopical findings in this study consisted of a decrease in the number of functional melanocytes and a reduction in the amount of melanin in the affected hypopigmented skin without any appreciable change in the shape, size and cytoplasmic activity of the melanocytes, or the number and length of dendrites. The transfer of melanosomes to keratinocytes appeared to be unaltered, except in one case where there was a focal disturbance in transfer in areas of marked spongiosis. The hypopigmentation thus appears to be due mainly to a reduction in the number of melanocytes and possibly a decrease in the production of melanosomes, although enhanced degradation of melanosomes in keratinocytes remains a possibility. The slight decrease in melanosomal size in the affected skin may also play some role in causing the hypopigmentation.

Although various microscopical abnormalities were present in the affected skin in nearly all cases under study, none of these findings alone would explain all the changes in the pigment cell or its product. The histopathological findings were entirely non-specific, as reported by Wells *et al.* (1960). Hyperkeratosis and parakeratosis were noted in only half the cases under study and were mild, thus it would seen unlikely that these abnormalities in the keratin layer play a significant role in the pathogenesis of the hypopigmentation. Two cases showed a moderate degree of exocytosis and spongiosis while the remainder exhibited some degree of intercellular oedema at the ultrastructural level. Intracytoplasmic lipid droplets were present in the majority of cases. The significance of these findings, particularly in relation to the hypopigmentation, is not clear, and requires further study. It should be pointed out, however, that autophagic melanosomal vacuole formation in the melanocyte is not a prominent feature of the cases under study and thus the condition differs from naevus depigmentosus in which melanosomal autophagy in melanocytes is frequently present (Jimbow *et al.*, 1975). The only significant finding in the dermis is a mild to moderate perivascular inflammatory infiltrate in some cases. Of interest are the presence of mild intercellular oedema, intracytoplasmic lipid droplets and perivascular inflammatory infiltrate in the control skin adjacent to the lesions. All these changes are of a milder degree than those seen in the lesional skin. The intercellular oedema is unlikely to be due to technical error. The involvement of the adjacent skin, therefore, may be analogous to that in psoriasis vulgaris where clinically completely normal-looking skin in interpsoriatic areas shows remarkably psoriasiform histological changes (Braun-Falco, 1976).

The cases under discussion differed clinically from patients with classical pityriasis alba in that they were predominantly adults and the condition was of a longer duration. Furthermore, the rash was generalized and symmetrical, usually over the neck and arms and much less frequently over the face. The lesions were generally neither scaly nor erythematous and were asymptomatic. Post-inflammatory hypomelanosis such as occurs after the clearing of papulosquamous disorders, secondary syphilis, drug eruptions, pityriasis versicolor, etc., was ruled out by the absence of a preceding rash. A subclinical affection by conditions such as pityriasis rosea was excluded by the long duration of the rash in the majority of these patients. It is possible, therefore, that the cases under discussion may represent a new entity of undetermined aetiology. A detailed histochemical and ultrastructural study of classical pityriasis alba would be useful for comparison, but this would be more difficult to perform because of the usual localization of lesions to the face in young patients.

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Pharmacology and treatment

Pharmacokinetic observations on dapsone in dermatitis herpetiformis

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SUMMARY

The pharmacokinetics of dapsone (DDS) and monoacetyldapsone (MADDS) following an oral dose of 150 mg DDS were studied in sixteen patients with dermatitis herpetiformis and seven normal subjects. No differences in DDS disposition were observed between the two groups. The maintenance dose of DDS for individual patients was not significantly correlated with jejunal biopsy morphology, DDS or MADDS half-lives, or the area under the plasma concentration-time curves for DDS or MADDS. DDS plasma protein binding was normal in patients and did not apparently determine the concentration of DDS in skin biopsies, for which the skin/plasma DDS concentration ratio was approximately unity. There was no undue representation of acetylator phenotype in the patient group and no correlation between maintenance dose and MADDS/DDS ratio was noted. The determinants of the maintenance DDS dose have not been found. This may relate to pharmacodynamic differences, but alternatively the concentration of oxidative metabolites rather than DDS or MADDS could be responsible for the therapeutic activity of DDS in dermatitis herpetiformis.

The dose of dapsone (diamino-diphenyl sulphone; DDS) required to control the rash of dermatitis herpetiformis varies in different patients from 50 mg weekly to 700 mg daily. The cause of this interpatient variability is unknown but clearly could be related either to pharmacokinetic differences between patients or to differences in the susceptibility of the disease to treatment in each patient. Some of the pharmacokinetic variables which could contribute to this difference in dose are reduced absorption of DDS due to an accompanying gluten sensitive enteropathy, intersubject differences in half-life and rate of metabolite formation and the acetylator phenotype. In this study some aspects of the pharmacokinetics of DDS have been investigated and related to the dose necessary to control the rash.

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PATIENTS AND METHODS

Pharmacokinetic studies were carried out in two groups of subjects according to a protocol approved by the ethics committees of Guy's and St Mary's Hospitals.

(1) Sixteen patients who had been suffering from dermatitis herpetiformis for a median period of 7·25 years (range 5 months to 23 years). All had IgA in the dermal papillae in uninvolved skin biopsies. In all patients the total plasma protein and albumin concentrations were normal. Some patients were taking a gluten-free diet (see Table 1). The daily DDS dosage ranged between 14·3 and 600 mg: JJ was in diet-induced remission and GS was a mild case not requiring DDS treatment. DDS was discontinued 3 days before the study in patients taking 100 mg/day or less and 7 days in those taking more than 100 mg/day to reduce the plasma concentration of DDS in these patients on chronic DDS therapy to low levels.

(2) Seven normal subjects (ages 24–36 years; weights 54–92 kg).

Subjects were fasted for 8 h before taking an oral dose of 150 mg DDS and they remained fasting for the first 4 h of the study. Venous blood samples were taken at 0 (pre-dose) 2, 3, 4, 8, 24 and 72 h into lithium heparin tubes in the majority of patients. The plasma was separated by centrifugation and stored at −20°. A full-thickness skin biopsy was taken at 4 h in the patients only and stored at −20°. A small intestinal biopsy was also taken in the dermatitis herpetiformis patients and the mucosal appearance recorded using a dissecting microscope.

Plasma concentrations of DDS and monoacetyldapsone (MADDS) were determined by high performance quantitative thin-layer chromatography as previously described (Ahmad & Rogers, 1980a). This method is specific and has a minimum level of detection for DDS

TABLE 1. Details of patients undertaking DDS pharmacokinetic study

Patient	Age (years)	Weight (kg)	Usual daily DDS dose (mg)	Disease duration (years)	Additional drug therapy (mg/day)
GR	32	78	100	7	—
DM	40	60	400*	9	Diazepam 10; phenytoin 300; oral disodium cromoglycate 400; carbamazepine 400
WZ	44	67	600	10	—
CF	65	50	200	6	—
VW	44	53	14·3*	15	Flurazepam 60
EE	54	94	100*	4·5	—
ZK	43	63	100	6	—
TE	47	49	40	23	Diazepam 15
GS	32	62·5	0*	0·5	—
CJ	55	58	10*	9	—
HH	51	72	100	4	—
JJ	47	70	0*	4	—
RC	30	54	80	7½	—
CC	38	54	100	23	—
MJ	63	92	33	9	Oral disodium cromoglycate 1500
AM	21	70	100	6	—
Median	44	61·3	90	7·25	

* Indicates gluten-free dietary regime.

(duplicate readings not differing by more than 10%) of 20 ng/ml. The coefficient of variation within assays ranged between 7·8% at 100 ng/ml to 3·5% at 1200 ng/ml.

The apparent first order rate constant of elimination for DDS from the plasma (k) was determined by linear regression of ln (plasma concentration) on time in the elimination phase. The plasma half-life was found from $T_{\frac{1}{2}} = 0{\cdot}693/\mathrm{k}$. The area under the plasma concentration, time curve (AUC) was determined using the linear trapezoidal rule with appropriate extrapolation for the infinite portion of the curve. Where DDS was present in the pre-dose plasma sample appropriate correction was made for the contribution of this to the total AUC.

DDS binding to plasma proteins was determined using Amicon Diaflo Ultrafiltration cones (Amicon Ltd, Amersham, Bucks) with estimation of DDS in the ultrafiltrate by quantitative thin layer chromatography as described by Ahmad & Rogers (1980b).

The weighed skin biopsies were thoroughly homogenized in 0·067 M pH 7·4 Sørensen phosphate buffer using an ultrasonicator (Polytron, Kinematica GmbH, Basel) and the DDS concentration then estimated in this extract by chromatography as above.

Additional studies were carried out in:

(1) Twelve patients with dermatitis herpetiformis who were acetylator phenotyped using dapsone by measurement of the concentration ratio MADDS/DDS at 3 h after an oral dose of 150 mg DDS. Slow acetylators were defined as having MADDS/DDS ratios less than 0·30 and fast acetylators as having ratios greater than 0·35 (Gelber *et al.*, 1971; Reidenberg *et al.*, 1973). Thus the acetylator phenotypes of twenty-eight patients with dermatitis herpetiformis were available for comparison with the normal population frequency of acetylator phenotypes.

(2) Plasma protein binding of DDS was determined in another four normal subjects giving a total of eleven measurements for this variate in normal subjects.

Non-parametric statistical methods were employed throughout. The Mann-Whitney U-test was used to contrast variates from two independent groups. Association between variates was determined by either Spearman's rank order correlation coefficient (r_s) or the biserial correlation coefficient (r_b) where appropriate. Corrections for ties were made as required. Fisher's exact test was used for comparison of observed and expected frequency of acetylator phenotypes.

RESULTS

Table 1 gives details of the patients studied. Tables 2 and 3 summarize the derived pharmacokinetic values for DDS and MADDS in patients and normal subjects respectively. No patients or volunteers suffered adverse effects related to participation in this study.

No correlation between AUC and maintenance DDS dose was found ($r_s = 0{\cdot}522$; $t = 2{\cdot}11$—not significant). Similarly no significant correlation was found with gut morphology ($r_b = 0{\cdot}02$). Wright & Das (1980) have suggested that the 'speed of absorption' of DDS was reduced in patients with dermatitis herpetiformis. 'Speed of absorption' was not based upon the absorption rate constant but upon standardization of plasma levels by multiplying the plasma concentrations at 2 and 4 h (only) after 50 mg DDS by a factor to normalize for body weight (weight in kg/60). These normalized values for their patients were found to be significantly lower than those of normals. The number of plasma concentrations determined during the absorption phase in our study was too few to allow accurate estimation of absorption rate contants. Using the standardization factor employed by Wright & Das (1980) the normalized peak plasma concentrations in our patients were median 2·88 μg/ml; mean 2·92 s.d. 0·76 μg/ml and in the normal subjects median 3·10 μg/ml mean 3·28 s.d. 0·67 μg/ml. There was no

TABLE 2. Pharmacokinetic values for DDS and MADDS with gut biopsy morphology in patients with dermatitis herpetiformis

	DDS			MADDS					
Patient	$k(h^{-1})$	$T_{\frac{1}{2}}$(h)	AUC (μg/ml h)	$k(h^{-1})$	$T_{\frac{1}{2}}$(h)	AUC (μg/ml h)	MADDS/DDS ratio	Skin DDS/plasma DDS ratio	Gut biopsy morphology
GR	0·034	20·4	53·7	0·022	31·5	8·6	0·16	0·98	Flat
DM	0·037	18·7	17·8	0·039	17·9	3·5	0·22	1·43	Fingers
WZ	0·043	16·1	73·4	0·030	23·4	20·4	0·28	1·01	Convoluted
CF	0·050	13·9	15·7	0·043	16·1	4·1	0·26	0·97	—
VW	0·021	33·0	57·2	0·019	36·8	5·0	0·20	1·03	Leaves
EE	0·043	16·1	28·6	0·040	17·5	25·6	0·89	1·09	Fingers
ZK	0·022	31·5	181·5	0·023	30·4	29·8	0·17	0·99	—
TE	0·028	24·8	82·9	0·025	27·1	21·6	0·25	1·07	Convoluted
GS	0·035	25·1	82·6	0·043	16·1	9·1	0·13	1·07	Convoluted
CJ	0·024	29·3	103·3	0·029	23·9	63·2	0·76	1·11	Fingers
HH	0·030	23·1	76·4	0·023	29·8	18·8	0·19	1·08	Fingers
JJ	0·029	24·2	63·5	0·029	23·6	7·1	0·11	1·15	Fingers
RC	0·034	20·3	51·6	0·035	19·7	24·8	0·48	1·16	—
CC	0·030	23·1	69·8	0·030	23·1	13·8	0·20	—	Flat
MJ	0·026	26·7	71·1	0·027	25·7	19·1	0·27	1·06	Flat
Am	0·018	38·5	66·6	0·029	23·9	9·0	0·13	1·09	Flat
Mean	0·032	24·1	70·3	0·030	24·2	18·2		1·09	
s.d.	0·009	36·3	6·7	0·008	6·0	14·4		0·11	
Median	0·030*	23·1*	68·2†	0·029†	23·8†	16·3†		1·08	

P—for difference between patients and normal subjects: * $0{\cdot}05 > P > 0{\cdot}02$. † Not significant.

TABLE 3. Pharmacokinetic values for DDS and MADDS in normal subjects

	DDS			MADDS			
	k (h^{-1})	$T_{\frac{1}{2}}$ (h)	AUC (μg/ml h)	k (h^{-1})	$T_{\frac{1}{2}}$ (h)	AUC (μg/ml h)	MADDS/DDS ratio
NS	0·022	32·2	58·2	0·023	30·1	36·0	0·61
MT	0·031	22·5	86·8	0·026	26·7	35·7	0·42
RA	0·027	25·5	66·4	0·024	28·5	10·6	0·17
IT	0·018	39·2	48·3	0·030	23·2	11·6	0·24
ER	0·024	28·8	60·9	0·021	32·6	15·9	0·25
SH	0·020	34·0	43·1	0·036	19·1	5·2	0·13
MJ	0·023	29·6	55·4	0·045	15·5	8·9	0·15
Mean	0·026	30·3	59·9	0·029	25·4	17·7	
s.d.	0·003	5·5	14·2	0·008	6·7	12·8	
Median	0·023	29·6	58·2	0·026	26·7	11·6	

significant difference between these values or in the AUC values between patients and normal subjects (Table 2) and thus we conclude that no difference exists in the DDS absorption rate or in its extent between our series of patients with dermatitis herpetiformis and normal subjects.

The median apparent first order elimination rate constant was 0·030/h giving a median half-life of 23·1 h in the patients which was just significantly different from the median elimination rate constant of 0·023/h and half-life of 29·6 h in the seven normal subjects. No significant correlation was found between the plasma half-life of DDS and the usual maintenance dosage in fourteen patients ($r_s = 0{\cdot}47$; $t = 1{\cdot}85$—not significant).

The protein binding of DDS in the sixteen patients varied between 69 and 80% (median 76%; mean 74%, s.d. 3%). No significant difference was found in this respect from a control population of eleven normal subjects in whom the DDS binding varied between 72 and 84% (median 76%; mean 77%, s.d. 4%).

The mean ratio of skin to plasma concentrations at 4 h was 1·09 with a coefficient of variation of 10%. All except one patient had a ratio very close to unity.

Nine of twenty eight patients were fast acetylators. In our twenty-eight patients, twenty-four were on maintenance dapsone treatment. The median daily maintenance dose in the eight fast-acetylators was 90 mg (mean 73, s.d. 33.8 mg), and that in the sixteen slow acetylators was 100 mg (mean 145·6, s.d. 150·6 mg). No significant correlation was found between the individual MADDS/DDS ratios and maintenance DDS dose. Similarly in the sixteen patients in whom MADDS concentration, time profiles were determined correlations were sought between the maintenance dose and the AUC and half-life of MADDS. The Spearman rank correlation coefficients were 0·425 and 0·481 respectively: neither of these values has stochastic significance. No significant difference was found between the patients and our normal subjects with respect to the absolute values of these variates.

DISCUSSION

This study shows that the overall absorption of DDS in patients with dermatitis herpetiformis as determined from the AUC was similar to that found in normal subjects and this did not correlate with the morphology of the jejunal biopsy or with the individual patients' maintenance dose. Alexander *et al.* (1970) administered ^{35}S-dapsone to patients and normal subjects and showed no significant differences in the amount of radioactivity absorbed in the two groups. Similarly the complete absorption of DDS in dermatitis herpetiformis was also demonstrated by Wright & Das (1980) using a colorimetric assay technique. They found no differences in the 24-h urinary DDS excretion between normal subjects and patients with dermatitis herpetiformis or coeliac disease. These authors further asserted, however, that the 'speed of absorption' of dapsone was reduced in patients with dermatitis herpetiformis. The implication of the study by Wright & Das (1980) is that the peak plasma concentration of DDS should be lower than that in normals (the adoption of the maximum observed plasma concentration rather than that at some arbitrary time increases the chance of finding a difference). This difference was not demonstrated in our group of patients and normal subjects. A similar finding is illustrated, without comment by the authors, in the radio-labelled studies of Alexander *et al.* (1970).

The pharmacokinetic behaviour of DDS and MADDS in patients with dermatitis herpetiformis conforms to that observed in normal subjects by ourselves and others (Gelber *et al.*, 1971; Glazko *et al.*, 1968). Similarly the protein binding of DDS is not apparently different in such patients from that found in normal subjects by ourselves and others (Biggs & Levy, 1971; Ahmad & Rogers, 1980b).

DDS is polymorphically acetylated in man. It has been shown that subjects who are slow acetylators of isoniazid have MADDS/DDS ratios less than 0·30 and that fast acetylators have ratios greater than 0·35 (Gelber *et al.*, 1971; Reidenberg *et al.*, 1973). The utility and accuracy of assignment of correct acetylator phenotype by this single sample dapsone test has been confirmed recently by Hanson, Melander & Wahlin-Boll (1981). Nine of twenty-eight patients (32·1%) were found to be fast acetylators. In a study of 135 Britons, Evans (1969) found a frequency of 38% of fast acetylators. This population of patients with dermatitis herpetiformis thus contains no undue proportion of fast acetylators ($P = 0{\cdot}74$; Two-sided Fisher exact test). This contrasts with the situation in systemic lupus erythematosus where the incidence of the slow acetylator phenotype is greater than would be expected in a random population (Drayer & Reidenberg, 1977).

The concentrations of DDS observed in the skin biopsies suggests that the ratio between skin and plasma is close to unity so that the plasma concentration may be a good indicator of skin DDS concentration, i.e. they belong to the same conceptual pharmacokinetic compartment. No data have been previously published providing the actual concentration of DDS in human skin but a similar skin/plasma ratio has been observed in mice (R.A.Ahmad, unpublished). The observed variation in plasma protein binding between patients is not apparently reflected by the skin concentration which is related to the total plasma DDS concentration. This suggests that plasma protein binding of DDS does not determine penetration in the skin. Alexander *et al.* (1970) found a uniform distribution of isotope in skin biopsies taken following administration of ^{35}S-dapsone. There was no evidence of concentration in specific areas of the autographs and they concluded that local concentration of the drug was not necessary for its therapeutic action. This is in contrast to the finding by Chatterjee & Podder (1957) of concentration of radio-labelled dapsone in the chronic cutaneous granulomata of leprosy. The action of DDS in leprosy is not, however, necessarily related to that in dermatitis herpetiformis.

The usual maintenance dose of these patients was not apparently related to the half-lives of DDS or MADDS or to the AUCs of these compounds or to the MADDS/DDS ratio. Some of the patients were also receiving concurrent drug therapy (Table 1) which could conceivably alter DDS disposition. Statistical analyses which omitted these patients did not change any of our conclusions concerning the pharmacokinetics of DDS or MADDS. The mean maintenance dose for fast acetylators was variable and not significantly different from that for slow acetylators. The strongest interpretation which can be placed upon this result, therefore, is either that the differences in maintenance DDS dose depend upon variable responses of the rash between patients (i.e. a pharmacodynamic variability) or else the therapeutic effect of DDS in dermatitis herpetiformis is due neither to the concentration of DDS nor MADDS. A similar conclusion was reached by Ellard *et al.* (1974) who found that the control of symptoms was not related to plasma dapsone or monoacetyldapsone concentration. Acetylation accounts for only about 60% of dapsone metabolism, the remainder being N-hydroxylated by hepatic microsomal enzymes (Israili *et al.*, 1973). Possibly these latter substances are responsible for the activity of DDS in dermatitis herpetiformis. Unfortunately these compounds are not available for clinical use and methods for their assay, apart from radio-labelling techniques, have not been developed so that correlation of clinical features with their concentration cannot be undertaken. It is, however, known that enzyme-inducing agents such as rifampicin accelerate DDS metabolism via this (inducible) oxidative pathway (Gelber, Gool & Rees 1975). This hypothesis could be tested in patients with dermatitis herpetiformis requiring concurrent treatment with inducing agents and those undergoing treatment with dapsone alone.

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Colchicine in the treatment of acute febrile neutrophilic dermatosis (Sweet's syndrome)

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SUMMARY

We describe three cases of Sweet's syndrome which showed very rapid improvement with colchicine.

Sweet's syndrome shows a massive polymorphonuclear leukocyte (PMN) infiltration in the dermis, with increased chemotactic activity of the peripheral blood PMNs in the acute stage (Oseroff *et al.*, 1981). We have suggested that colchicine would be effective in Sweet's syndrome as in other diseases in which PMNs play a crucial role (Matsumura & Mizushima, 1975; Hazen & Michel, 1979) and we noted a rapid regression of the lesions with this therapy in one case (Suehisa & Tagami, 1981). We now report its effectiveness in three additional cases of Sweet's syndrome.

REPORT OF CASES

The clinical and laboratory findings are summarized in Table 1. All three cases had typical skin lesions; dusky-red, tender, swollen plaques or nodules histologically showing massive infiltration with a variable mixture of PMNs and mononuclear cells. In addition to the typical signs, oral aphthosis was noted in two cases, and in case 3 an erythema nodosum-like lesion was found on the left lower leg which histologically showed the features of thrombophlebitis. In case 2 a skin biopsy specimen from the dusky-red plaque on the dorsum of the right hand showed the features of 'vasculitis', i.e. fibrinoid degeneration of the dermal vessels with peripheral PMN infiltration and nuclear debris.

Treatment with oral colchicine was started with 1·5 mg daily and clinical improvement appeared within 2–5 days in all patients. Cases 2 and 3 were able to stop taking the drug after 7 days, and in case 1 the dosage of colchicine was gradually reduced to 0·5 mg daily over 3 weeks to prevent recurrence. In case 2 the effectiveness of colchicine was confirmed in two different attacks.

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TABLE 1. Summary of three cases

	Cases		
	1	2	3
Age/Sex	38/F	35/M	44/M
Clinical features			
Sites of eruptions	Eyelid Cheek Forearm	Dorsum of the hand Forehead Cheek	Dorsum of the hand and finger Right inner canthus
Recurrence of attack	+	+	+
Fever	+	−	+
Symptoms of preceding infection	+	+	−
Arthralgia	−	−	+
Aphthosis	−	+	+
Unusual features		Vasculitis in HE section	Thrombophlebitis of lower leg
Laboratory studies * before and † after colchicine			
WBC (% of neutrophils)	6600 (74)* 6400 (64)†	5700 (61)* ND	7000 (49)* 6000 (57)†
ESR (mm/h)	54*→14†	42*→ND	15*→7†
CRP	+ +→ −	+ +→ND	− →ND

ND—Not determined

Increased erythrocyte sedimentation rate (ESR) and positive C-reactive protein (CRP) findings returned to the normal range after treatment with colchicine. Leukocytosis with neutrophilia was not noted in any case at the start of the therapy, possibly because of early presentation.

DISCUSSION

As stated by Sweet (1979), not all cases presenting typical skin lesions showed other features such as fever and neutrophilia.

Although some of our cases showed additional findings which have been described only rarely in previous reports on Sweet's syndrome, such as leukocytoclastic vasculitis (Shapiro, Baraf & Richheimer, 1971; Dobbeleer *et al.*, 1980), thrombophlebitis (Sweet, 1964) and oral aphthosis (Sweet, 1964), we diagnosed these cases by the characteristic morphology of the skin lesions and the history. The histological picture of obvious 'vasculitis' in case 3 resembles that of the Arthus type reaction, which has been reported by Sweet (1968). Oral aphthosis in cases 2 and 3 and thrombophlebitis in case 3 may indicate a link between Sweet's syndrome and Behçet's disease, both of which are characterized by an overreactive state of PMNs (Matsumura & Mizushima, 1975).

Colchicine has long been used in the management of gouty arthritis and more recently in familial Mediterranean fever, Behçet's disease (Matsumura & Mizushima, 1975; Miyachi *et al.*, 1981) and necrotizing vasculitis (Hazen & Michel, 1979). Although colchicine affects various leukocyte functions *in vitro*, the only leukocytic activity affected by a low plasma level of colchicine is the inhibition of PMN chemotaxis (Wallace, Omokoku & Ertel, 1970). Recently, Miyachi, Danno & Imamura (1981) reported the *in vivo* suppressive effects of colchicine on the active Arthus reaction in rabbits. They suggested that colchicine inhibits vascular injury by interfering with directional chemotaxis of PMNs to the lesional sites.

Only in case 3 did we investigate the chemotactic activity of the peripheral blood PMNs before and after the treatment with colchicine. As reported by Oseroff *et al.* (1981), the increased chemotactic activity towards the culture filtrate of *Escherichia coli* returned to control levels after colchicine therapy (unpublished data).

The improvement of our cases with colchicine may be due to a similar mechanism to that which occurs in Behçet's disease and necrotizing vasculitis. Further trials of this drug in Sweet's syndrome are warranted.

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Brief Communication

The level of phospholipase A_2 activity is raised in the uninvolved epidermis of psoriasis

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SUMMARY

The activity of phospholipase A_2 was measured in uninvolved psoriatic epidermis and normal epidermis. The mean phospholipase A_2 activity in uninvolved psoriatic epidermis, expressed as nmol fatty acid released per mg protein per h, was raised compared with normal epidermis ($7{\cdot}88 \pm$ s.e. $1{\cdot}20$ vs $2{\cdot}18 \pm$ s.e. $0{\cdot}38$; $P < 0{\cdot}01$). This observation is, we believe, the first demonstration of an abnormality in the uninvolved untreated psoriatic epidermis which could account for the pathology of the disease.

Psoriatic epidermis incorporates relatively less [^{14}C] acetate, in proportion to [^{32}P] orthophosphate or [^{14}C] glycerol, into phospholipids than normal epidermis (Summerly, Ilderton & Gray, 1978). This could be due to an increased rate of removal of fatty acids by a phospholipase, or a decreased rate of incorporation of fatty acids by an acyl transferase, or a combination of these two processes. Yardley & Summerly (1981) showed that this abnormal phospholipid synthesis can be corrected *in vitro* when anti-inflammatory steroid is added to the culture medium, and *in vivo* after application of steroid cream. It is now well established that anti-inflammatory steroids act by inhibiting phospholipase A_2 (Flower, 1981). We therefore chose to test the hypothesis that phospholipase A_2 activity is raised in psoriatic epidermis.

PATIENTS AND METHODS

Using a Davies dermatome, skin biopsies measuring approximately 10 × 10 mm were taken from uninvolved areas in nine patients suffering from psoriasis using ethyl chloride spray anaesthesia, each biopsy being at least 50 mm from a lesion. In some patients, biopsies were taken from lesions. Biopsies were also taken from nine normal volunteers. Epidermis was separated from dermis by heating at 50°C for 3 min and homogenized in an all-glass tissue grinder. Phospholipase A_2 in the homogenate was estimated by measuring the rate of release of labelled fatty acid when the epidermal homogenate was incubated at pH 8·5 with 2-[^{14}C]-oleoyl-1-palmitoyl phosphatidyl choline, essentially according to the method of Freinkel & Traczyk (1980).

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RESULTS

The mean phospholipase A_2 activity of lesional epidermis, expressed as nmol fatty acid released per mg protein per h, was similar to that of normal epidermis (Table 1). However, the mean phospholipase A_2 activity of uninvolved psoriatic epidermis was significantly raised compared with normal epidermis ($P < 0{\cdot}01$).

DISCUSSION

Since the anatomy of lesional epidermis is quite different from that of normal and uninvolved psoriatic epidermis, it is difficult to make a direct comparison between lesional and normal epidermis: the values for lesional epidermis are included merely for completeness. The values that appear in Table 1 for lesional epidermis are greatly influenced by the high protein content of the lesion, doubtless due in large measure to serum exudate. However, the demonstrated difference in the phospholipase A_2 activity between uninvolved psoriatic epidermis and normal epidermis is valid because of the anatomical similarity of these two tissues. Phospholipase A_2 activity showed no obvious dependence upon age, sex and severity of psoriasis, nor with the site of biopsy.

TABLE 1. Phospholipase A_2 activity in uninvolved and lesional psoriatic epidermis and normal epidermis. Units of enzyme activity are n moles fatty acid released per mg protein per h

	Normal epidermis	Uninvolved psoriatic epidermis	Lesional psoriatic epidermis
n	9	9	6
Mean	2·18	7·88	3·05
s.e.mean	0·38	1·20	1·14

The finding that phospholipase A_2 activity is increased in the uninvolved psoriatic epidermis is of fundamental importance for two reasons. Firstly, enhanced activity of this enzyme could lead to increased concentration of free arachidonic acid thus providing the potential for increased concentrations of inflammatory eicosanoids. Secondly, the other product of phospholipase action, lysophospholipid, is also reported to be inflammatory and vasoactive (Vadas, 1982).

Our finding has been foreshadowed to some extent by a report (Plummer *et al.*, 1978) that the level of free arachidonic acid is elevated in the uninvolved skin of 8-methoxypsoralen-treated patients with psoriasis. The fact that elevated levels of free arachidonic acid, which is known to be the rate limiting factor in the production of inflammatory eicosanoids (Flower, 1981), may exist in clinically univolved psoriatic epidermis, suggests that post-arachidonic control mechanisms operate in the uninvolved psoriatic epidermis to limit the production of inflammatory mediators.

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Society Proceedings

Proceedings of the 225th Meeting of the Netherlands Society for Dermatology and Venereology, Groningen, 8 May 1982

PRESIDENT: PROF. E.VAN DIJK
HOST: PROF. A.H.KLOKKE

SUMMARIES OF PAPERS

Prevalence of dermatoses of the hands and arms in the Netherlands

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Most patients in occupational dermatology clinics present themselves with the advanced stages of eczema/dermatitis of the hands. To detect risk factors associated with the milder, earlier stages, dermatitis of the hands was studied in the general population. Prevalence rates of hand dermatitis were obtained from two population-based samples (in two different geographical areas) consisting of a total of 3140 adults in the Netherlands. Between these samples/areas the crude prevalences among males were not significantly different. Age seemed to be important, but adjustment for age suggested that there was a difference between the two samples/areas, for which other factors might be responsible. The males could be classified in six major occupational groups. Age tended to be associated with occupation. Also the distribution of the males over the occupational categories was different in the two areas. To disentangle the relative importance of age, occupation and area, a step-wise logistic regression of dermatitis-prevalence was performed on these factors. The results showed that occupation was a significant factor. The age factor was not significant before and after correction for occupation. Also the contribution by the factors area of residence and age-occupation interaction was insignificant.

Three major occupational groups could be identified in which prevalences of hand dermatitis were relatively high: chemical industry 14·4%, metal industry 10·7%, and building/construction 7·1%. In all these categories more irritant (toxic) than allergic dermatitis was observed. For further investigation of occupation related risk factors, studies are planned in these high-prevalence occupations. In the construction industry a cross-sectional study with internal case-control design is well on its way. Preliminary results confirm that irritant (toxic) rather than allergic factors are important in the early stages of hand dermatitis, especially among bricklayers and plasterers.

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Chronic urticaria: intolerance for aspirin and food additives and relationship with atopy

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During a period of 10 years, patients with chronic urticaria were investigated and classified according to well defined criteria. Excluding some small groups of patients with urticaria due to allergy, rare kinds of physical urticaria and urticaria due to internal diseases, about half of the remaining patients appeared to be suffering from the four main kinds of physical urticaria: urticaria factitia, delayed pressure urticaria, cold urticaria and cholinergic urticaria.

Of 386 patients tested, 25% appeared to have a positive oral provocation test to aspirin. The highest percentages were found in patients with idiopathic urticaria, delayed pressure urticaria and cholinergic urticaria. In 17% of patients with physical urticaria positive tests were found, whereas 34% of patients with idiopathic urticaria and angio-oedema were positive. Aspirin intolerance was not found in any of the forty-three patients with cold urticaria.

Oral provocation tests with azo dyes and benzoates were performed in 271 patients. Positive reactions were found in approximately 5–10% of these patients. In the group of patients with physical urticaria they were less frequent however than in those with idiopathic urticaria and angio-oedema.

In a scatter diagram there was a significant clustering of aspirin intolerance with food additives intolerance. A correlation between aspirin intolerance and atopy could not be demonstrated.

A novel treatment of pyoderma gangrenosum by intestinal decontamination

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Recent reports have pointed to the role played by Gram-negative bacteria of the digestive tract in ulcerative colitis and rheumatoid arthritis, two diseases often associated with pyoderma gangrenosum. We were able to quantify the bacterial flora of the digestive tract of two patients with longstanding severe pyoderma gangrenosum. Estimation of faecal flora revealed *Escherichia coli* in a concentration of 10^8 bacteria per gram faeces (normal values $< 10^4$/g). In both patients previous treatments with salazopyrine, clofazimine, prednisone and azathioprine had not been successful. Elimination of the aerobic Gram-negative rods (*E. coli*) by selective decontamination of the digestive tract (van der Waay, 1979) resulted in a complete healing of skin lesions within 4 weeks. Similar findings were reported by Jablonska (1963).

To explain the foregoing we hypothesize a cross-antigenicity between aerobic Gram-negative rods and skin antigen. This theory and the possibility of a non-specific immunological event need further study.

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Basal cell naevus syndrome: immune status, etretinate therapy and etretinate hepatitis

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Three patients with basal cell nevus syndrome (BCNS) and multiple basal cell carcinomas were treated with oral etretinate (Tigason), approximately 1 mg/kg daily.

The number and size of the carcinomas were noted and photographed. Clinical suspicions were confirmed by histology as much as possible.

The immune response was investigated by assessing the reaction to the primary test immunogen *Helix pomatia* haemocyanin, lymphocyte stimulation tests with recall antigens and mitogens, PHA-induced lymphocyte cytotoxicity and the semi-quantitative DNCB-test.

Two patients were treated for 7 months, one patient for 4 months. The number of lesions suspected to be basal cell carcinomas smaller than approximately 10 mm in diameter decreased in all three patients. Larger carcinoma generally did not change in size. New basal cell carcinomas were not observed during the period of treatment. Within 4 months after discontinuation of treatment in all three patients recurrences were noted of carcinomas which seemed to have disappeared clinically during treatment.

One patient developed a biopsy-proven hepatitis. Circumstantial evidence (recurrence of the hepatitis after re-introduction of the drug, no serological evidence of a viral hepatitis) suggested that the hepatitis was caused by etretinate. The initial course of etretinate in this patient was discontinued after 4 months because of the hepatitis. The number of carcinomas, which had decreased considerably clinically during treatment, had returned to pretreatment level 7 weeks later and increased in the following months. This massive and speedy recurrence may be due to a change in the pharmacokinetics of etretinate because of the hepatitis.

Conclusions. (1) Based on our results, treatment of basal cell carcinomas with etretinate alone cannot be recommended. (2) Etretinate may be effective in the prevention of growth of basal cell carcinomas. (3) A therapeutic or preventive effect of etretinate on basal cell carcinoma is obstructed by etretinate-induced hepatitis, possibly due to altered pharmacokinetics. (4) The immune response *in vitro* of these three patients with basal cell naevus syndrome and multiple basal cell carcinomas was not found to be abnormal in those tests performed. The response to the DNCB-test *in vivo* was abnormal. No consistent changes in the *in vitro* immune response were noted during etretinate treatment.

The effect of thalidomide in the treatment of lupus erythematosus

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Eleven patients with severe chloroquine-resistent chronic cutaneous lupus erythematosus were treated with thalidomide. Seven patients responded with a complete remission and two patients improved significantly. One patient did not respond to therapy and another had to be withdrawn from the trial because of side-effects. In six patients a maintenance dosage was necessary. Side-effects were minor and reversible.

The need is stressed for optimal measures to avoid pregnancy in fertile women.

Does history condition the present?

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This question, if applied to dermatology, is not the obvious rhetorical one which one would expect. Medical mycology and dermatopathology provide us with examples in which this question cannot easily be answered in the affirmative.

Early dermatology was modelled upon these disciplines which were the offspring of dermatologists. So far, between 1848 and 1982, more than twenty handbooks on dermatopathology have been written, all of them except one by dermatologists.

These facts are not reflected in the present situation, as both disciplines tend to fade away from the dermatologists' daily life. In this meeting a case of favus is presented in a 67-year-old woman who for years underwent immunofluorescent studies on her scarred scalp before the much older discipline of medical mycology was applied for successful examination. Similarly, a tropical patient with tuberculoid leprosy was mistaken for granuloma annulare, due to the fact that the clinical picture was not correlated with the microscopical one.

Most dermatologists nowadays send biopsies for examination to general pathologists, and are satisfied to receive a written report like the one they receive from the clinical pathologist about the leukocyte count. They are not in a position to obey the maxim of Unna (1894) who in the introduction of his 'Treatise on Histopathology of Skin Diseases' made the point that dermatologists have the duty 'to carry out clinical examination with an eye trained in microscopy and on the other hand to look through the microscope with a keen clinically experienced eye'.

Interrelating the clinical picture with knowledge of the microscopic features of the same lesion provides for continuous postgraduate education in the consulting room. The lack of facilities for the dermatologist to do so deprives him of one of the roots upon which his skill should firmly rest.

The rebirth of dermatopathology which nowadays we are witnessing may help to bring dermatopathology back into the daily life of the dermatologist.

CASE REPORTS

Traumatic swellings in Weber-Christian disease, due to multiple protease inhibitor deficiencies

E.Bleumink and A.H.Klokke

Female, aged 27 years.

History and diagnosis. For many years the patient had complained of painful lumps on the arms and legs, later spreading to the mammae, gluteal area and back, which disappeared, sometimes discharging fluid, leaving depressed scars. Apart from these nodules the patient was found to develop enormous swellings after relatively minor trauma. No fever or arthritis was found to occur. No internal pathology could be detected. A slight leukocytosis without eosinophilia and a decrease of C3 could be detected during the active phase. Histopathological pictures (of biopsies from nodules) were compatible with nodular panniculitis in its first acute inflammatory stage (Weber-Christian stage I).

Studies of mediator system. Biochemical examination of the clotting, fibrinolysis, kallikrein and complement systems of this particular patient showed, besides a slightly depressed C3 level, multiple plasma protease-inhibitor deficiencies (Table 1).

TABLE 1. Serum levels of plasma protease inhibitors

	Patient	Normal values
Alpha-l-antitrypsin	0·278–0·400	2·170 ± 0·720
Anti-trypsin activity	0·074–0·229	1·251 ± 0·266
Anti-chymotrypsin activity	0·000–0·172	0·701 ± 0·332

Phenotyping of alpha-l-antitrypsin revealed a Pi ZZ homozygote. In family members MZ-heterozygotes were found. Anti-chymotrypsin deficiencies were also detected in family members.

Comment. In addition to the findings of Rubinstein (1977) and Warter (1972) the present results strongly indicate that protease inhibitor deficiencies may predispose to or aggravate the course of panniculitis.

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Woolly hair naevus with triangular hairs

M.F.HASPER AND A.H.KLOKKE

Female, aged 22 years.

History. Since birth she had noticed a 'strange patch' on the scalp but she had no other physical complaints.

Examination. Above the left ear was a sharply demarcated patch, approximately 5 cm in diameter, of scalp hair that was different in shape and consistency from the surrounding normal straight, fair hair. The hair in the affected area was darker in colour and the hair itself was thinner, softer and curly.

Complete physical examination revealed no abnormalities.

Light microscopy of affected hairs. There was irregular distribution of pigment, with torsions and narrowings, a normal anagen-telogen ratio, and several dysplastic hair roots.

Scanning electron microscopy of affected hairs. There was decrease of the hair diameter. In several places the structure of the cuticule was disturbed. There was twisting of the shafts, with torsion of the hair around the long axis at irregular distances (90–360°). There were longitudinal grooves, often several together on the same hair shaft (Fig. 1). There were ovoid, triangular and irregular formed cross sections (Fig. 2).

Investigations. No abnormalities in blood cell count, ESR, liver function, renal function and urinalysis were noted.

Treatment. No treatment was given, as the lesion did not cause any problems to the patient.

Comment. Our patient had no linear naevus elsewhere on the body, as is often reported in the literature. The triangular cross section and canaliform dystrophy, as reported in spun glass hair

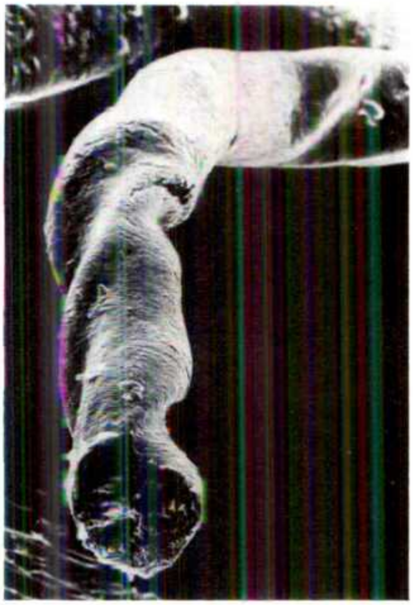

FIGURE 1. Woolly hair naevus. Electron microscope photograph of an affected hair.

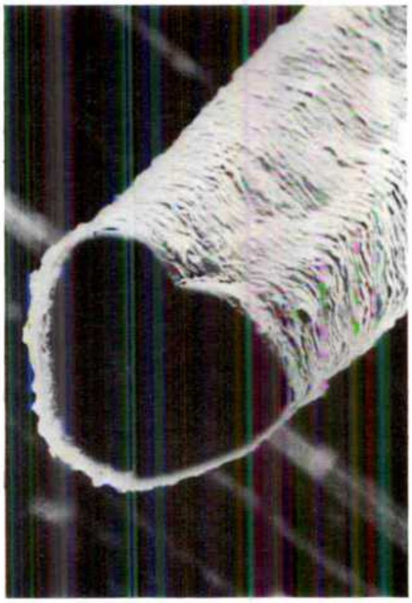

FIGURE 2. Electron microscope photograph of a cross section of an affected hair.

('uncombable hair'), have not, to our knowledge, previously been reported in patients with woolly hair naevus.

Leprosy, borderline-lepromatous, and cytomegalovirus infection

J.A.KRUYT, A.H.KLOKKE, H.W.ROENHORST AND T.H.THE

Male, aged 53 years.

History. This Indonesian patient has been living in the Netherlands almost continually for 30 years. He left Indonesia in 1951. At that time he seems to have had a white spot on his right cheek. In 1979 he visited Indonesia for several weeks. In August 1980 a coronary-bypass operation was performed in Geneva. In September 1980 the patient noticed red, elevated spots on his face which spread to the trunk.

Examination. The face showed red-brown, confluent plaque-like lesions. Some similar lesions were found asymmetrically on the trunk apart from some lightly hypopigmented macules. The nerves were not thickened. There were no functional disturbances. There was no loss of sensitivity, except on the lateral side of the lower left leg.

Investigations. Screening of haematological, renal and hepatic functions revealed no abnormalities, except a slightly elevated ESR (28 mm/h). W. R. negative. Immunoglobulins normal. Chest X-ray normal. Antibodies cytomegalovirus (February 1982)—CMV-EA 1:2560, CMV-LA 1:10.240. CMV-induced lymphocyte transformation tests: low reactivity of patient's lymphocytes against CMV-virion antigens and virtually negative reactivity against CMV-cell membrane antigens. Lymphocyte reactivity against different mitogens (PHA, Con A and PWM): abnormal low response, especially against Con A. Mixed lymphocyte culture: normal response.

Histology. A biopsy from a lesion on the face shows large histiocytic infiltrates with an admixture of lymphocytes. By Wade-Fite staining numerous bacterial rods, partly in clusters, are seen. Also granular rods (± 30%) and granules (± 10%) are seen.

Treatment. Rifampicin, 600 mg daily, isoprodian, two tablets daily (one tablet contains isoniazid 175 mg, protionamid 175 mg, dapsone 50 mg), pyridoxine 20 mg daily.

Comment. It is unlikely that this patient acquired his leprosy in the Netherlands. It is possible that a lepromatous infection, possibly acquired more than 30 years ago, was activated because of a cytomegalovirus infection. This possibility is supported by our immunological studies showing an active CMV-infection possibly related to the defective cellular immunity against CMV infected cells (Schirm, Roenhorst & The, 1980). Also a depressed cellular immune status was found, especially a low responsiveness against Con A, which may correspond also to a secondary immunosuppressive effect presumably due to the active CMV-infection. We do not know whether our patient acquired a primary CMV-infection by blood transfusion or reactivation of a latent CMV-infection during the coronary bypass operation in 1980. A CMV-infection is known to be instrumental in depressing the immune response (ten Napel & The, 1980) and may be related to the immunocompromised situation of homosexuals threatened by opportunistic infections (Editorial, 1981a, b).

In the treatment of bacilliferous leprosy, experience has been gained by treatment with at least one bactericidal drug in combination with more than one bacteriostatic drug (Klokke, 1982). It is hoped that in this case of lepromatous leprosy all treatment can be stopped after two or three years of this regimen.

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Subacute cutaneous lupus erythematosus with polyneuropathy

F. VAN OOSTVEEN AND P.C. VAN VOORST VADER

Female, aged 53 years.

History. Since 1974 this patient has had a skin disorder, initially on the face but since 1977 also on the trunk and extremities. There are spontaneous remissions and exacerbations. Sunlight aggravates the disorder. The patient complains of a loss of sensitivity of both feet. Rheumatological symptoms are not present.

Examination. The trunk, especially the back, the limbs and the face present a symmetrical erythematous slightly raised eruption consisting partly of papules, partly of annular and polycyclic lesions, which become scaly and disappear without scarring (Fig. 3).

Investigations. ANA positive, speckled type (1:10^2–10^6). Anti-ds-DNA negative. LE-cell test: a sporadic LE-cell. Cryoglobulins positive (IgG and IgM). HB a.g. and a.l. negative. Rheumatoid serology, lues serology negative. Leukocytes, thrombocytes, immunoglobulins, renal function, urine, complement (C3 and C4) normal.

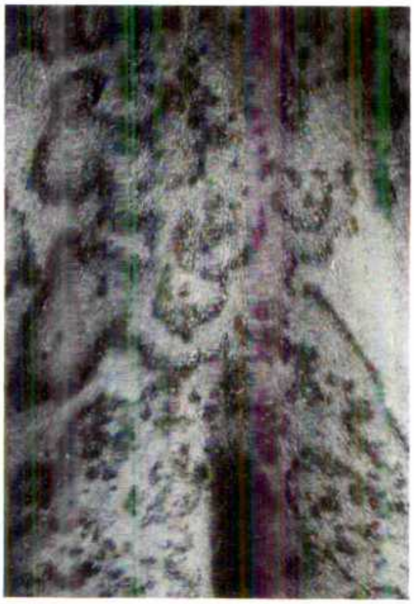

FIGURE 3. Figurate erythematous and papular eruption on the back.

Histology. Epidermis with hydropic degeneration in the basal layer. Subepidermal oedema and perivascular round cell infiltration.

Immunofluorescence. In one lesion, fine, discontinuous granular deposits of C3 and C1q along the dermo-epidermal junction and C3 deposits in some subepidermal vessel walls were observed. The normal skin showed fine granular deposits of IgM, C3 and C1q along the dermo-epidermal junction.

Neurological investigations. The existence of a polyneuropathy was confirmed by electromyography.

Treatment. The antimalarial chloroquine was started in March 1980 and resulted in a complete remission of the cutaneous and neurological symptoms. In January 1981 this treatment was stopped. In February 1981 the neurological symptoms recurred as well as a few cutaneous lesions. Treatment was recommenced.

Comment. Sontheimer, Thomas & Gilliam (1979) first distinguished a distinctive subgroup of lupus erythematosus called subacute cutaneous lupus erythematosus. The cutaneous symptoms, which are a striking feature, consist of papular and/or annular-polycyclic erythematous lesions which become scaly and heal without scarring. Diagnosis may be difficult because of a negative investigation for anti-nuclear antibody (ANA) and negative or dubious findings in the immunofluorescence. Investigation for anti-Ro antibody in ANA negative patients may be useful.

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Partial lipodystrophy ('facies Voltarien') and hypocomplementaemic nephritis

E.J.KRAAI AND W.VAN SON

Female, aged 23 years.

History. This patient is the only member of her family to have ascetic features with a striking depression of the surface of the cheeks. The patient seems to have had these features since the age of 5 years. At that age she also suffered from infections of the middle-ear. At the age of 20 she developed a nephrotic syndrome after an infection of the throat. A kidney biopsy was found to be compatible with the early stage of a membrano-proliferative glomerulonephritis. Moreover the patient was shown to have a marked depression of the C3-level in the peripheral blood. The C4-level was also found to be depressed.

Examination. The face showed ascetic features with a depression of the surface of the cheeks. Subcutaneous fat was not palpable. A biopsy could not be obtained (Fig. 4).

Immunofluorescence. The normal skin from the extensor side of the lower arm showed marked deposits of C3c in the subepidermal vessel walls. Immunoglobulin, C1q and C4 deposits were not seen.

Investigations. Creatinine clearance—lowered (50–70 ml/min). Urine—albuminuria (3·2–6·6 g/24 h), hematuria, leucocyturia. ANA positive, homogeneous. C3 0·267 (1·250 ± 0·201). C4 0·198 (0·325 ± 0·107). Haemolytic complement CH 100 57 U (97 ± 15). Haemolytic complement alternative pathway (AP 100) 7·6 U (> 6·5) C3 nephritic factor not present.

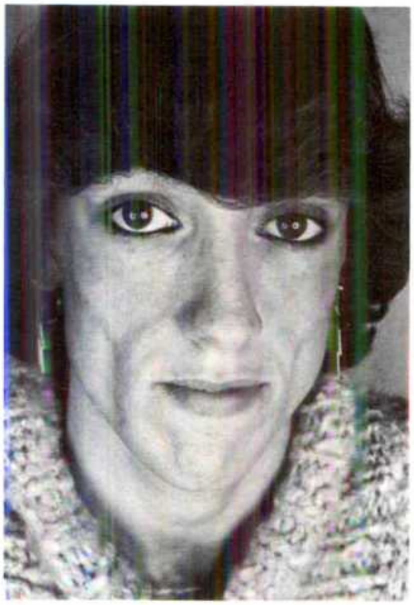

FIGURE 4. 'Facies Voltarien' in partial lipodystrophy.

Comment. Partial lipodystrophy is seen mainly in the face of women and usually appears in childhood or during adolescence. At the time of appearance of the lipodystrophy a lymphocytic panniculitis (Peters & Winkelmann, 1980) may be found. In about 80% of the patients with partial lipodystrophy, a lowered C3-level in the serum is found. In about 25–50% of the patients with partial lipodystrophy, a nephritis develops some 5–20 years after the appearance of the lipodystrophy. Almost all patients who develop a nephritis have an abnormal complement profile.

Our data indicate activation of the classical pathway of complement in our patient.

In about 30% of the patients with hypocomplementaemic membrano-proliferative glomerulonephritis the lowered serum level of C3 is explained by the presence of C3-nephritic factor, an IgG auto-antibody, which is capable of activating the complement by the alternative pathway.

Partial lipodystrophy is also known to be associated with diabetes mellitus and is thought to be associated with an increased risk of infections.

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Linear IgA dermatosis

A.J.VELDERS, M.C.J.M.DE JONG AND P.C.VAN VOORST VADER

Male, aged 35 years.

History. For 4 years this patient had suffered from a pruritic eruption. The individual lesions healed spontaneously in about 2–4 weeks. Mucosal lesions were not noticed.

Examination. Sparse solitary vesicles or small bullae were seen, mostly on an erythematous base, disseminated over the entire skin.

Histology. Below a normal epidermis, blister formation was seen. In the blister granulocytes and some eosinophils were present. The dermis showed perivascular round cell infiltrates.

Immunofluorescence. Direct IF of lesional and normal skin both show linear deposits of IgA along the basement membrane. Deposits of other immunoglobulins are not seen. In direct IF using guinea-pig oesophagus as substrate linear deposits of IgA (titre 1:80), not of IgG, are found along the epithelial basement membrane.

Using bullous pemphigoid serum on a biopsy of vesiculo-bullous skin of the patient, the B.P. antigen was found to be present in the floor as well as in the roof of the blister, which suggests splitting of the epidermal basement membrane at the level of the lamina lucida (Fig. 5).

Investigations. HLA-typing B8 DR3. Anti-reticulin antibodies, anti-gluten antibodies, epicutaneous tests with potassium iodide, tests for circulating immune complexes (PEG-precipitation test, C1q-binding test) negative. Vit B12, folic acid normal. Xylose resorption test normal. Jejunal biopsies were not performed.

Treatment. Dapsone, 100–150 mg daily only partially suppressed the lesions. Sulfapyridine, 500 mg daily, was no more effective than dapsone.

Comment. Bullous pemphigoid serum was used to localize the level of split formation in the epidermal basement membrane. The level appeared to be at the lamina lucida as is usual in bullous pemphigoid. Consistent with this finding is the absence of evidence of a malabsorption syndrome in the limited number of tests performed. Intestinal abnormalities do not seem to be frequent in patients with linear IgA dermatosis (Lawley *et al.*, 1980; Leonard *et al.*, 1981; Marsden *et al.*, 1979). However, the HLA-haplotype is similar to the typing seen in many patients with dermatitis herpetiformis. This HLA-haplotype seems to be related to a defective Fc-receptor function (Lawley *et al.*, 1981).

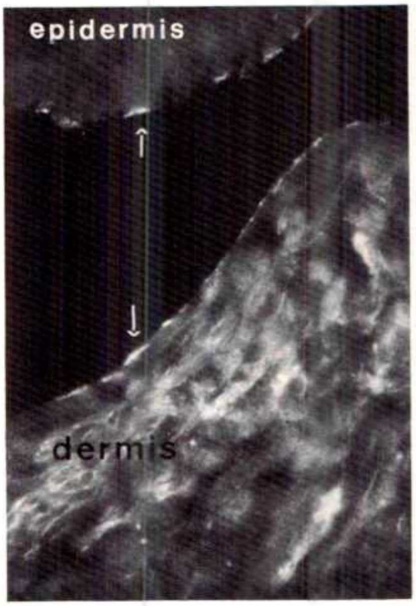

FIGURE 5. Linear IgA dermatosis. Cryostat section of a skin lesion treated with bullous pemphigoid serum (1st layer), followed by incubation with goat anti-human IgG fluorescein conjugate (2nd layer).

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Omenn syndrome: familial reticulo-endotheliosis with eosinophilia and combined immunodeficiency

A.J.VELDERS, W.KUIS, H.A.VAN DIJK, S.POPPEMA, J.D.ELEMA, A.H.KLOKKE AND P.C.VAN VOORST VADER

Male, aged 1½ years.

History. In the first months of life this baby showed a skin eruption of the scalp suggestive of a seborrhoeic dermatitis. From the age of 1 year a widespread pruritic skin disorder developed with fever, lymphadenopathy, anaemia and eosinophilia. Topical steroids were not effective. From the age of 14 months chronic diarrhoea was present. This baby was the first child of healthy, seemingly unrelated parents. In their respective families similar diseases were unknown.

Examination. Sharply demarcated erythematosquamous disseminated lesions were seen on the head, trunk and limbs. On the legs especially the lesions were more plaque-like and erythematous without scaling. Three small violaceous nodules were observed on the arms and legs. (Figs 6 and 7).

Histology. Biopsy from a nodule showed acanthosis, an oedematous papillary layer and in the lower part of the dermis a dense infiltrate consisting of histiocytes, lymphocytes, eosinophils and mast cells. There was no epidermotropism.

Biopsy from an erythematous lesion showed a sparse perivascular infiltrate of lymphocytes and histiocytes.

Biopsy of a lymph node showed a picture suggestive of dermatopathic lymphadenopathy with enlargement of the paracortical areas with an increase of histiocytic cells besides many lymphocytes with irregular nuclei, large blasts with basophilic cytoplasm, eosinophils and mast cells.

In the follicles the stain for ecto-5-nucleotidase was hardly visible compared with a very prominent staining in a normal control lymph node. Bone marrow biopsy was normal.

Electron microscopy. No Langerhans cells were found in the biopsy from a cutaneous nodule and in the lymphnode.

Investigations. Anaemia, leukocytosis (WBC 20,000/mm^3) with 30% eosinophils. Liver and kidney functions normal. Total protein and albumen decreased. No albuminuria. X-ray of chest, skull and pelvis normal. Liver and spleen scan normal.

Immunological investigations. During hospitalization of the patient the IgG and IgA serum levels dropped to abnormal values, possibly because of loss of globulins through the skin. IgM levels remained normal. IgE levels were not elevated. Lymphocyte transformation tests by mitogens and recall antigens repeatedly resulted in only a limited stimulation. Phagocytosis,

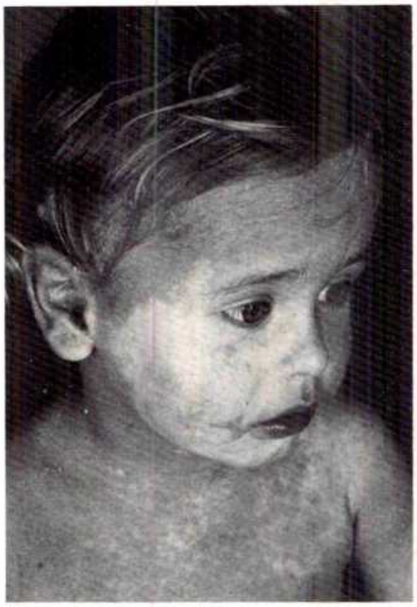

FIGURE 6. Omenn syndrome. Seborrhoeic dermatitis-like lesions on the face.

opsonizing potential and granulocyte chemotaxis were normal. Percentage T-lymphocytes peripheral blood normal. Percentage B-lymphocytes peripheral blood decreased (one sample). On the surface of peripheral blood lymphocytes ecto-5-nucleotidase appeared to be present only in a very limited amount compared to a control. The lymphocytes of the parents showed a normal amount of ecto-5-nucleotidase.

These investigations suggest the presence of a combined cellular and possibly also humoral immunodeficiency in this patient.

Treatment and progress. Shortly after hospitalization the patient developed erythroderma with severe pruritus. Septicaemia occurred several times. The lymphadenopathy increased markedly. At the age of 20 months the patient died of 'toxic shock'.

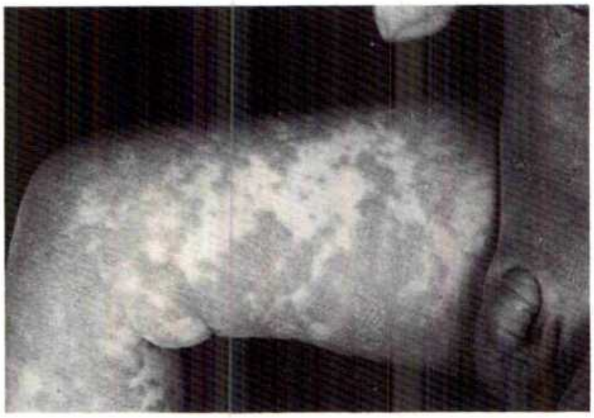

FIGURE 7. Erythematous slightly elevated lesions on the leg.

Autopsy. The infiltrative abnormalities were restricted to the skin and the lymph nodes. There was lymphocytic depletion of the thymus.

Comment. Omenn (1965) first described a familial, probably autosomal recessive, syndrome consisting of an immunodeficiency, a chronic skin eruption resembling the Letterer-Siwe type of histiocytosis X, hepatosplenomegaly, eosinophilia and histiocytic infiltration of the lymph nodes. Whether the immunodeficiencies in this syndrome result from the infiltrative disorder, or should be considered as the primary abnormality, is unknown.

Cohen *et al.* (1980) observed a decreased presence of ecto-5-nucleotidase on the peripheral lymphocytes of one of these patients. A clear interpretation of this observation is not possible.

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Cutaneous malignant epidermotropic lymphoma, possibly of monocytic origin

E.J.Kraai, S.Poppema, P.C.van Voorst Vader, H.M.G.Doeglas and M.C.J.M.de Jong

Male, aged 53 years.

History. In July 1980 the first cutaneous lesion was observed on the right upper leg. Subsequently an increasing number of new lesions spread over the entire skin.

Examination. (September 1980.) This revealed an apparently healthy man apart from the fact that disseminated over the entire skin, infiltrated erythematous and erythemato-squamous, partly annular plaques were observed. The primary lesion on the right upper leg had grown into an ulcerating tumour. A tumour was seen on the tongue. Abnormal lymph nodes were not palpable. Liver and spleen were of normal size.

Investigations. WBC 5,800/mm^3 with normal leukocyte differentiation. A monocytosis in the leukocyte differentiation was never observed. In April 1981, using alpha-naphthylbutyrate esterase staining of the mononuclear leukocytes of a Ficoll-Hypaque prepared buffycoat, 60% of the cells appeared to be monocytes versus 15 ± 7·4% in controls. Chest X-ray, abdominal CT scan, liver and spleen scan and marrow biopsy were normal.

Histology. Cutaneous biopsies showed a dense infiltration of the epidermis with blast-like cells. In tumours, infiltration of the dermis was also seen. A biopsy of the tumour on the tongue also showed an epidermotropic infiltrate with these atypical cells. An inguinal lymph node showed an active paracortical area, where tumour cells similar to those in the skin were found sporadically.

DNA-histogram. In imprint preparations of skin lesions an abnormal increase in the triploid and tetraploid range was observed.

Enzyme histochemistry. The tumour cells showed a paranuclear area with granular acid phonuclear leukocytes and natural killer cells (OKM 1 and 2) and Ia-like antigen (OKI 1) were all negative.

Immunohistology. Anti-Ig antibodies, monoclonal anti T-lymphocyte antibodies (OKT 1, 3, 4, 5, 6, 8 and 11) and monoclonal antibodies against peripheral blood monocytes, polymorphonuclear leukocytes and natural killer cells (OKM 1 and 2) and Ia-like antigen (OKI 1) were all negative.

Electron microscopy. The cytoplasm of some of the atypical cells contained several electron-dense lysosomes. There was no striking indentation of the nuclei. Langerhans granules were not observed.

Treatment and progress. After an initial remission with COP-courses (cyclophosphamide, oncovin, prednisone) the disease was progressive despite chemotherapy with methotrexate with leukovorin rescue, bleomycin and a podophyllin derivate and addition of adriamycin to a COP-course. There was an enormous growth of some of the tumours. Eventually cerebral involvement developed and the patient died in August 1981.

Autopsy. Multiple skin tumours. Tumour in left testicle. Large numbers of tumour cells in the cerebro-spinal fluid.

Comment. Enzyme histological, immunohistological and electron microscopic investigations virtually exclude a T-cell lymphoma and suggest that the tumour cells might be of monocytic origin. Acute monocytic leukaemia produces cutaneous and mucous membrane involvement. Initial cutaneous involvement and a delayed leukaemic appearance of acute monocytic leukaemia has been described (Burg *et al.*, 1978). The epidermotropic infiltrate of the Pagetoid reticulosis of Woringer-Kolopp is supposed to contain T-lymphocytes as well as histiocytes.

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Correspondence

Scalp surface lipid

Sir, Saint-Leger and Leveque (*British Journal of Dermatology* (1982) **106**, 669) describe a method for the measurement of skin surface lipid on the scalp. We agree that the Lipometre technique is suitable for this type of measurement, but feel that the method described in the paper is imprecise and contains a serious error.

Due to the inherent symmetry of the integument, validation of sebum excretion rate studies has been performed by comparison of measurements taken from the left and right sides of the body (Cunliffe, Kearney & Simpson, 1980). We have performed left/right studies of sebum production using a Lipometre and 6 mm coarse-ground sapphire sampling plates in two series of fourteen and twenty-seven subjects (to be published). In the first series the scalp was prepared for measurement by cutting the hair close to the scalp with scissors as described by Saint-Leger and Leveque (1982). The casual level was measured on the left and right sides of the temporal region of the scalp with a correlation coefficient $r = 0{\cdot}75$ ($P < 0{\cdot}01$). Lipid was allowed to accumulate for 3 h and resampled at the same sites to give a value for sebum excretion rate which correlated similarly ($r = 0{\cdot}68$, $P < 0{\cdot}05$). These correlations although significant were not of the same order as corresponding left/right correlations found on forehand skin ($r = 0{\cdot}88$, $P < 0{\cdot}001$; Cunliffe, Kearney & Simpson, 1980) or on scapular back skin ($r = 0{\cdot}86$, $P < 0{\cdot}001$; Simpson, unpublished observations). The reason for these relatively poor correlations seems to be due to poor preparation of the scalp. The 'follicular pattern' which is unobtainable by the method of Saint-Leger and Leveque and is considered to be an essential starting point for sebum excretion rate studies (Strauss & Pochi, 1961; Cunliffe & Shuster, 1969; Cunliffe, Kearney & Simpson, 1980), may be achieved if the hair is cut by scissors and then shaved close to the scalp followed by 'blotting' with absorbent tissue. In a study of twenty-seven subjects with scalps prepared in this way a high left/right correlation ($r = 0{\cdot}91$, $P < 0{\cdot}001$) was achieved for a 3 h sebum excretion rate. Casual level measurements are impossible with this technique as shaving removes a variable amount of surface lipid.

Photometric methods for the measurement of skin surface lipid require the establishment of a partition coefficient between the skin surface and the sampling plate. We believe that the method described by Saint-Leger and Leveque contains a serious sampling error due to the failure of close approximation of the sampling plates to the scalp.

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Annular vasculitis in sarcoidosis

Sir, The recent report by Branford *et al.* of an annular vasculitis occurring in sarcoidosis (*British Journal of Dermatology* (1982) **106**, 713) resembles very closely a patient who presented to this hospital in 1981.

Case Report. A 30-year-old lady had a 2-week history of arthralgia, erythema nodosum and erythematous, annular plaques over both wrists. She had recently had several episodes of what she felt was a flu-like illness but has been otherwise previously fit. There were no symptoms or signs of eye disease.

Routine biochemistry and blood picture were normal but the ESR was over 100 mm/h. Chest X-ray showed bilateral hilar lymphadenopathy with clear lung fields. Further investigations showed a normal ASO titre and negative throat swab. Viral studies indicated a recent influenza A infection not thought to be related to her present problem. Serum and urinary calcium were normal, HBsAg and autoantibodies were negative and protein electrophoresis showed increased α_2 globulins only. C_3 was raised at 2668 mg/l (normal range 840–1652) as were PEG precipitable complexes at 187.2 µg/ml (normal range < 100). Lung function revealed normal volumes but with impaired gas transfer consistent with sarcoid. Mantoux 1/1000 was negative. A biopsy from the lesions on the wrists showed leucocytoclastic vasculitis and a subsequent Kveim test was positive. The serum angiotensin converting enzyme (ACE) was raised at 62 nmol/ml/min (normal range 16–52).

She was give only symptomatic treatment with aspirin and over the next few months her skin lesions cleared spontaneously leaving only post-inflammatory hyperpigmentation. The ESR became normal and the hilar lymphadenopathy disappeared.

The clinical history, the positive Kveim (false positive Kveim tests are rare, Poole 1982) and the raised angiotensin converting enzyme activity are strong evidence for a diagnosis of sarcoid in this patient. There are many similarities to the patient described by Branford *et al.* (1982). Both patients had similar clinical findings with an acute onset of the disease, markedly elevated ESR and raised α_2 globulins and a rapid and spontaneous improvement.

Circulating immune complexes have been shown in sarcoid (McI. Johnson *et al.*, 1980) and there is evidence in our patient for their presence. When they have been detected in sarcoid this has usually been associated with a high ESR (Geraint James, 1979).

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Immune responses in chronic dermatophyte infections

SIR, We read with great interest the excellent paper by R.J.Hay and G.Shennan on immune responses in chronic dermatophyte infections (*British Journal of Dermatology* (1982) **106**, 191–198).

It might be of interest in this context to mention that the main conclusions on hyporeactivity of lymphocytes in patients with *T. rubrum* infections and in patients with chronic dermatophytosis are in accordance with our own findings (Kaaman, Petrini & Wasserman, 1979). Furthermore, we observed a discrepancy between skin and lymphocyte reactivity, i.e. most patients with chronic infections were anergic to purified trichophytin in skin tests, while their lymphocytes responded to the same antigen. We concluded that a partial dysfunction of the cell-mediated immune response could be responsible for chronic dermatophytosis. Our recent studies on T-cell subpopulations indicate a relation between the severity of the disease and a decreased proportion of T-helper cells with concomitant increase of T-suppressor cells. Further studies to clarify these problems are in progress. For those interested in this research area we add some pertinent references.

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T. KAAMAN
B. PETRINI
J. WASSERMAN

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British Journal of Dermatology (1983) **108,** 127–128.

Book Reviews

Dermatology for the House Officer. Peter J.Lynch. (1982) Baltimore/London: Williams and Wilkins. Pp. 277. Price U.S. $9.95.

This handy book is one of the series 'For the House Officer'. In the preface the author states that this is a working book, written for medical students and house officers who find that dermatology must be learned by caring for patients. It is designed to be carried to the clinical setting where it can be quickly used to establish a list of differential diagnoses. There have been a number of text books based on the problem-orientated approach but most have failed, probably by trying to include too much. This, however, succeeds admirably. The author does not attempt to cover the whole of dermatology but concentrates on the relatively small number of diseases which comprise the very large proportion of cases seen by the dermatologist. In his extensive experience, he finds that fifty dermatological conditions account for 95% of all office visits for skin disease. These are given under their main presenting features together with a further fifteen included because of their seriousness, contagiousness or treatability.

The first section deals very adequately with basic principles of skin diseases. The next deals with problem-orientated diagnoses listed under such headings as the vesiculo-bullous diseases, skin coloured papules and nodules, yellow lesions, vascular reactions, etc. The final section deals succinctly with common problems in differential diagnosis and the last two chapters are general signs of systemic disease and specific signs of systemic disease. Each section has a short but comprehensive bibliography and there are appendices of major reference books, atlases and recent reference monographs.

The vast clinical experience of the author is evident. His description of diseases is lucid and short and, in spite of careful search, I found nothing with which I disagreed, including his inclusion of keratoacanthoma in the section of squamous carcinoma. This book should be readily available in the library of all busy general hospitals, and those who are considering the possibility of becoming dermatologists will find it stimulating and invaluable. This review copy is now in the library of the busy general hospital in which I work.

RENWICK VICKERS

Lymphoproliferative Diseases of the Skin. Edited by M.Goos and E.Christophers (1982) Berlin, Heidelberg, New York: Springer Verlag. Pp. 296. 149 Figs. Price U.S. $45.70.

A very successful International Symposium on Cutaneous Lymphoma held in Kiel in February, 1981 forms the basis of this volume which includes the clinical and investigative communications presented at the meeting.

For many, particularly in Europe, Kiel is the centre of the lymphoma world; thus numerous valuable contributions from Professor Lennert's group, particularly relating to the biology of T and B lymphocytes and the immune system, are encompassed in the first chapter of this book. Further chapters discuss pathogenesis of lymphoma, morphology, immunopathology and clinical aspects, and a separate chapter is devoted to the controversial subject of pagetoid reticulosis.

While the application of new techniques, amply discussed in this text, is gradually producing a logical classification of lymphoma and solving many individual questions, it is interesting to examine the progress of the co-operative study groups whose investigations are detailed in the final chapter. Mycosis fungoides, the dermatologists' own lymphoma, is a rare disease. The co-operative studies of several European countries are compiling much needed statistical data relating to staging, prognosis and treatment. Results of this work are not prominent in the British dermatological literature and for this reason the final chapter on Lymphoproliferative Diseases of the Skin is noteworthy.

The 'proceedings' of a Symposium inevitably varies in quality, depending on the individual contributors. Nevertheless Dr. Goos and Professor Christophers must feel satisfied that their editorial effort has produced a logical and interesting account of the advances in cutaneous lymphoma up to 1981.

D.M.MacDonald

Progress in Diseases of the Skin. Volume 1. Edited by Raul Fleischmajer (1981) New York: Grune and Stratton. Pp. 280. Price U.S. $39.50.

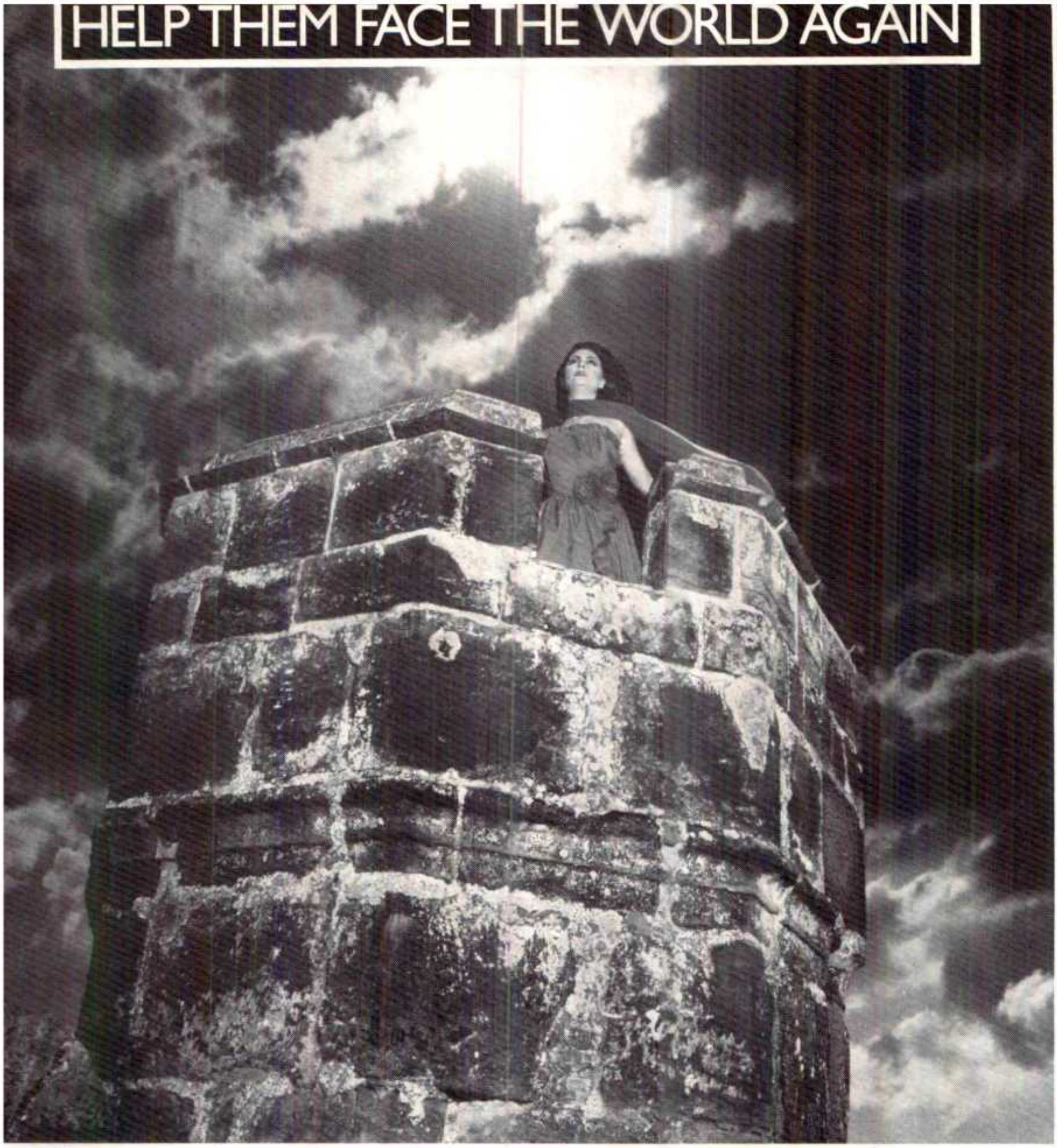
Raul Fleischmajer, professor of dermatology at the Mount Sinai Medical Centre, New York, is an eminent and highly respected clinical and scientific researcher in the field of collagen and connective tissue. His editorial task in producing volumes entitled 'Progress in Skin Disease' is far from easy. What is the significance of 'Volume 1'? Will later volumes update the contents of the first, or will different areas of progress be discussed? Progress in medical science, including dermatology, is currently very rapid. Consequently inevitable delays in book publication compounded by delays in reviewing would suggest that much of the data would be outdated by the time that the review is read.

Individual chapters include contributions on keratinization, Langerhans cells, prostaglandins in immunological responses, lysosomes, immune complexes, collagen, lymphokines, dermoepidermal junction, T lymphocyte-epidermal interaction, porphyrias, allergic vasculitis and retinoids—an impressive list and very topical.

I was very familiar with the content of some of the chapters, and thus was able to recognize that considerable progress had been made in that area since the time of writing. Furthermore, in writing of progress, essential background, often venerable, has of necessity been included. Other chapters were unfamiliar ground, with much new information to be gleaned. I remained conscious however that all this new refreshing information of progress carried me through only to about 1980.

Professor Fleischmajer has certainly selected exciting areas of progress that are currently in vogue, and as none of us are experts in everything, one cannot help but be further informed by reading this thoroughly digestible book whose every chapter is contributed by an expert.

D.M.MacDonald



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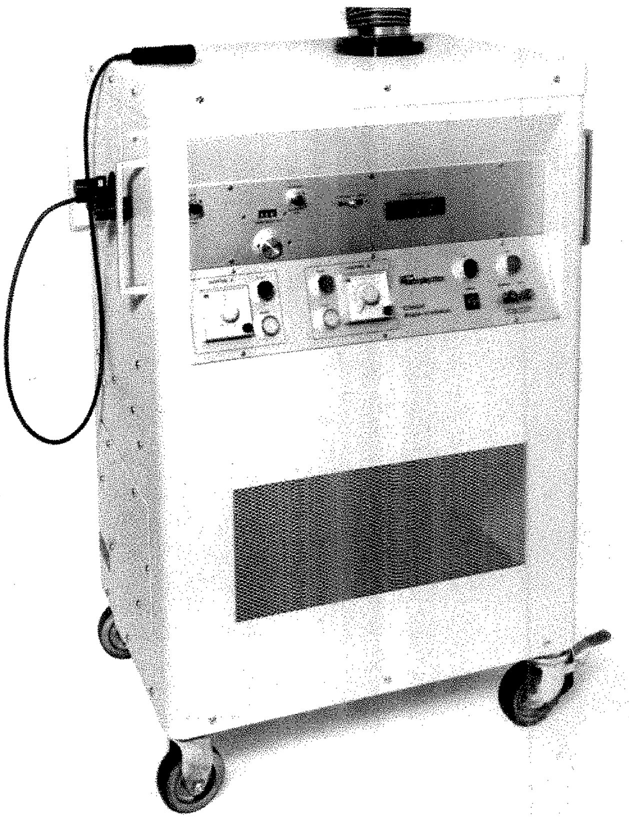

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microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm^3	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
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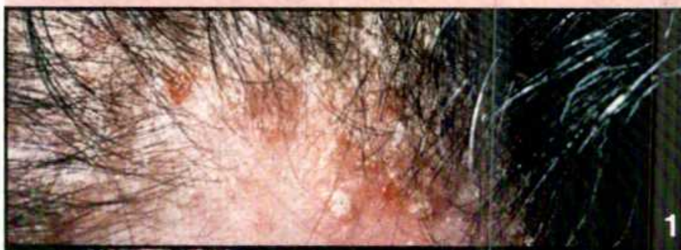
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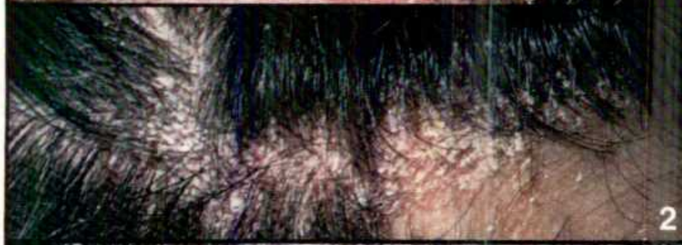
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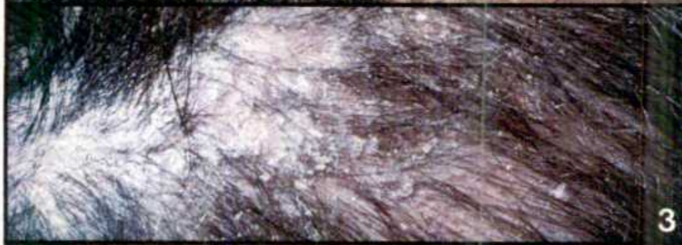
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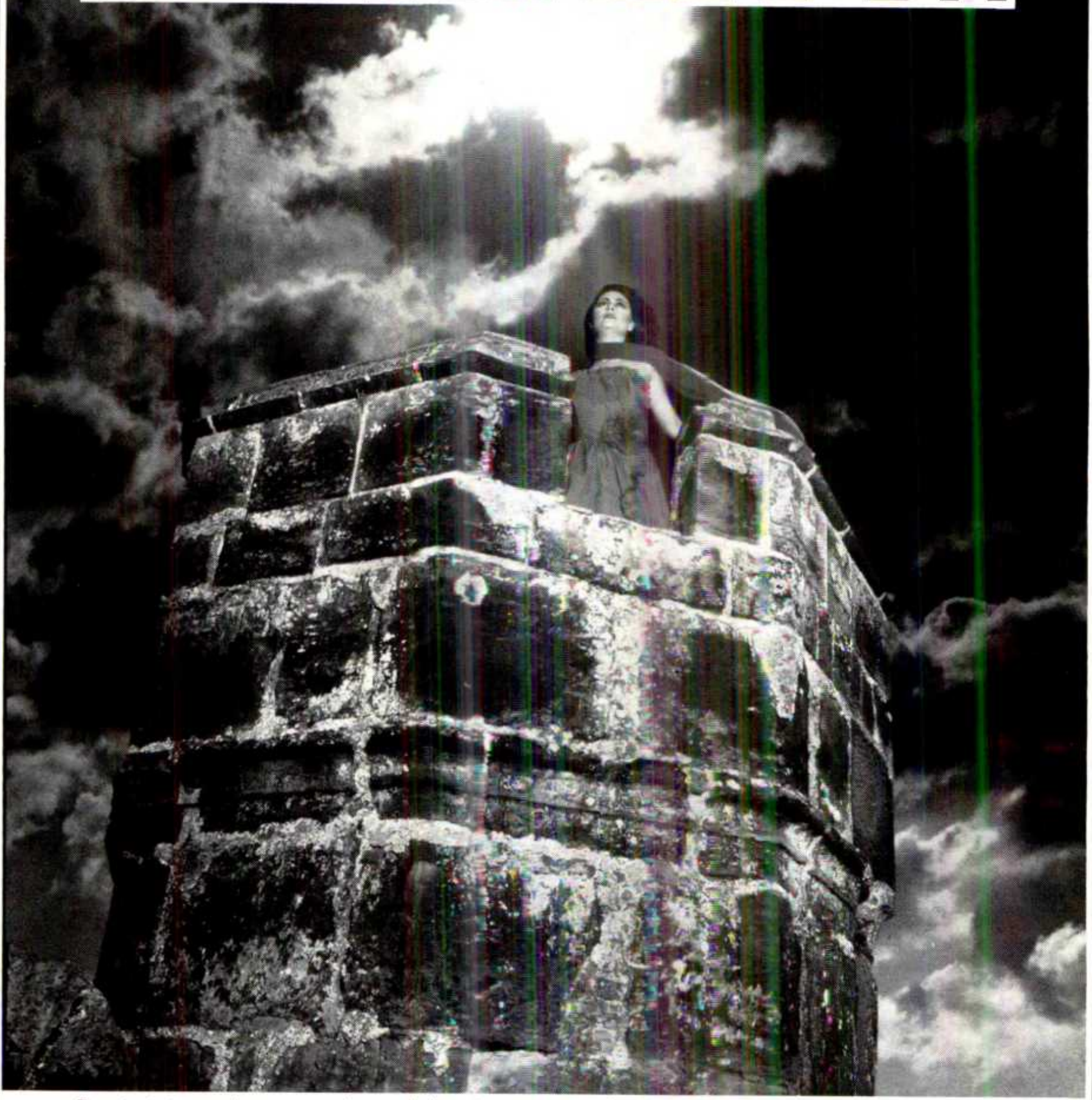

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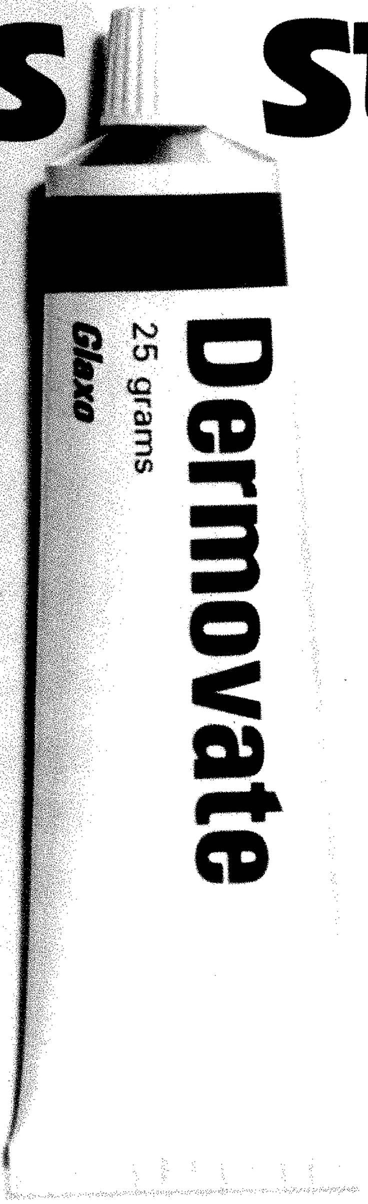

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Fry L. Br Med J 1982; 284:1093–10

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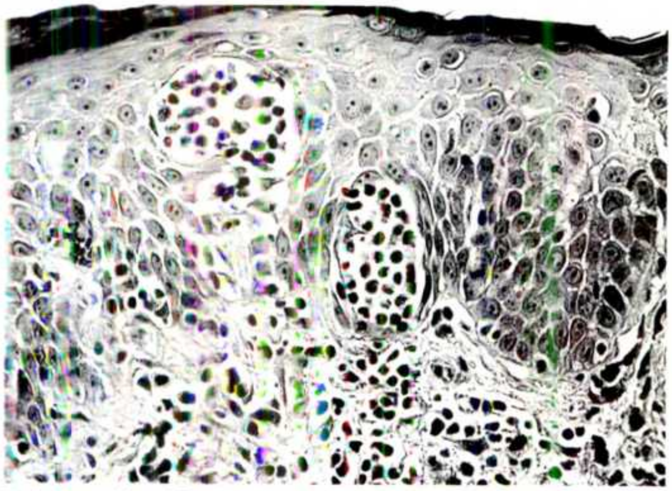
G. Burg, O. Braun-Falco

Cutaneous Lymphomas, Pseudolymphomas, and Related Disorders

With the collaboration of H. Kerl, L.-D. Leder, C. Schmoeckel, H. H. Wolff
With the assistance of M. Leider as editorial consultant

1982. Approx. 56 color and 150 black-and-white figures in 600 separate illustrations, approx. 26 tables. Approx. 550 pages.
Cloth DM 360,– (approx. US $ 150.20)
ISBN 3-540-10467-4

Contents: Introduction. – Methods of Study of Lymphocytes. – Morphology of Cells. – Enzyme Cytochemistry: Diagnostically Relevant Facts. – Demonstration of Immunologic Markers for the Identification of Lymphoid Cells in Cutaneous Infiltrates. – Patterns of Cellular Infiltrates in Cutaneous Lymphomas. – Classification of Malignant Non-Hodgkin's Lymphomas: Pathology, Hematology, and Dermatology. – Small Lymphoid Cell Lymphomas with Band-like T Cell Pattern (Mycosis Fungoides Type). – Small and Mixed Small and Large Lymphoid Cell Lymphomas with Nodular B Cell Pattern (Reticulosis Type). – Large Lymphoid Cell Lymphomas with Diffuse Non-B, Non-T Cell Pattern (Reticulo- and Lymphosarcoma Type). – Cutaneous Manifestations of Burkitt's Lymphoma. – Cutaneous Manifestations of Hodgkin's Disease. – Leukemic Lymphomas/Leukemias. – Diseases of the Mononuclear-Phagocyte System: Tumors and Tumor-Like Disorders. – Cutaneous Pseudolymphomas. – Miscellaneous Diseases Clinically Resembling or Related to Cutaneous Lymphoms or Pseudolymphomas. – Subject Index. – References.



Typical intraepidermal Pautrier microabsesses filled with mononuclear cells of various shapes and sizes. Note slight spongiosis of the epidermis.

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British Medical Journal, 1978, **2**, 84.

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British Journal of Dermatology (1983) **108**, 129–138.

Clinical and Laboratory Investigations

Functional and phenotypic analysis of isolated human Langerhans cells and indeterminate cells

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SUMMARY

In the mixed skin cell lymphocyte culture reaction (MSLR), epidermal cells induce the proliferation *in vitro* of peripheral blood lymphocytes. Using monoclonal antibodies (MCAB) OKT6 specific for Langerhans cells among epidermal cells and an anti HLA-Dr, we analysed which epidermal cells are necessary in MSLR in man. Inhibition and reduction of the proliferative responses in MSLR conducted respectively with 'anti HLA-Dr and complement' and 'OKT6 and complement'-pretreated epidermal cells suggested that not only Langerhans cells but also other HLA-Dr bearing cells play a major role. Immuno-electron microscopic studies of either HLA-Dr (+) or OKT6 (+) enriched and depleted epidermal cells suspensions: (1) were well correlated with results in MSLR; (2) indicated that Langerhans cells express both T6 and HLA-Dr determinants while indeterminate cells express only the HLA-Dr. No other epidermal cells were found to be either OKT6 or HLA-Dr (+). The data strongly suggest that not only Langerhans cells but also OKT6 (−) HLA-Dr (+) indeterminate cells play a major role in lympho-epidermal interactions.

The existence of immunological reactions between epidermal cells (EC) and lymphocytes (L) has been suggested by microscopic observations in various skin disorders characterized by a lymphocytic infiltration at the dermo-epidermal junction or within the epidermis (Edelson, 1980; Schmitt, 1981). A new tool for the investigation of lympho-epidermal interactions may be provided by the *in vitro* mixed skin cell lymphocyte culture reaction (MSLR), where EC are able to induce the *in vitro* proliferation of lymphocytes. This MSLR has been demonstrated in animals and in man between not only allogeneic but also autologous cells (Beucher & Saurat, 1979; Braathen & Thorsby, 1980; Faure *et al.*, 1982; Morhenn, Engelman & Farber, 1978) and is in many ways similar to the one-way mixed lymphocyte culture reaction (MLR) (Tanaka & Sakai, 1979).

Recent data support the hypothesis that epidermal cells expressing the HLA-Dr-Ia like

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antigens are necessary for the response of mononuclear cells in MSLR (Braathen & Thorsby, 1980; Stingl *et al.*, 1978a). Different types of epidermal cells have been reported to express the HLA-Dr specificity: Langerhans cells (LC) (Klareskog *et al.*, 1977; Rowden, Lewis & Sullivan, 1977; Stingl *et al.*, 1978b), indeterminate cells (IC) (Rowden, Phillips & Lewis, 1979) and perhaps keratinocytes in various pathological conditions (Lampert, Suitters & Chisholn, 1981; Mason, Dallman & Barclay, 1981; Tjernlund, 1981). Furthermore, LC are actually known to express a differentiation cell membrane antigen, named T-6 antigen, that may be revealed by the monoclonal antibody OKT-6 (Fithian *et al.*, 1981) thereby allowing their distinction from monocytes-histiocytes (Murphy *et al.*, 1981).

The aim of the present study was to determine the respective role of OKT6 reactive and HLA-Dr positive cells in the stimulation process induced by epidermal cells in MSLR. The depletion or enrichment of epidermal cells in the various subsets considered was obtained either through complement mediated inactivation by the appropriate monoclonal antibody or after separation of the specifically reactive cells on immunoglobulin coated plates. In parallel, immunoelectronmicroscopic studies using monoclonal antibodies specific for the T6 or the HLA-Dr determinants were conducted with epidermal cell suspensions, in order to characterize at the ultrastructural level the phenotype of the cell subpopulations involved in MSLR.

METHODS

Cells

Epidermal cells. Normal human EC were obtained from healthy volunteers from fresh surgical skin specimens. Skin was obtained from different locations (arms and forearms). Single cell suspensions were prepared from the epidermis as described previously (Eisinger *et al.*, 1979; Faure, Eisinger & Bystryn, 1981). Briefly, skin samples were freed of fatty tissue with scissors and washed three times with Hanks' balanced salt solution (Gibco, Glasgow, U.K.) with antibiotics. Discs of tissue were cut from the epidermal side of each specimen, washed in 0·02% EDTA (Merck, Darmstad, F.R.D.) and the pieces incubated in 0·25% trypsin (1:250, Difco, Chicago, Ill. U.S.A.) at 4°C. After 12–15 h the tissue was transferred to a fresh dish and the epidermis was detached from the dermis with fine forceps. Isolated epidermal samples were pooled in trypsin/EDTA and individual cells were teased apart with forceps and gentle pipetting. The process was repeated until the stratum corneum and an undetermined, but small number, of superficial keratinocytes adhering to the stratum corneum remained in the dish. The suspended cells were filtered through sterile gauze, washed three times, concentrated by centrifugation and resuspended in RPMI-1640 (Eurobio, Paris, France) supplemented with 10% heat inactivated AB human serum, 2 mM L glutamine and antibiotics (complete medium).

Lymphocyte isolation. Peripheral blood (PB) lymphocytes were obtained from healthy volunteers by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density centrifugation (Boyüm, 1976) of fresh defibrinated venous blood. Cell viability was in every case higher than 95% as judged by the trypan blue exclusion test.

Monoclonal antibodies

Two monoclonal antibodies (MCAB) were used:

(1) OKT6 MCAB (Ortho Pharmaceutical Corp., Raritan, NJ, U.S.A.) has previously been

shown to react with 70% of human intrathymic lymphocytes and among EC, specifically with LC (Fithian *et al.*, 1981; Murphy *et al.*, 1981).

(2) anti HLA-Dr MCAB, named BL2 was produced by J. Brochier (INSERM U 80, Hôpital E. Herriot, Lyon, France). Antibody specificity was shown to be identical to that of monomorphic anti HLA-Dr. It precipitates with material from B cell membranes showing two bands (Mol. wt 28,000 and 33,000), belongs to the IgG_{2b} isotype and is able to fix complement.

Complement-mediated lysis of cell suspensions with MCAB

Incubation of 2×10^6 EC in complete medium was made with either 0·2 ml of OKT6 MCAB (diluted 1:200) or 0·2 ml of anti HLA-Dr MCAB (final dilution 1:20) at 37°C for 45 min. Cells were then washed twice. Fresh rabbit complement (C′) was then added at a final dilution of 1:10 and incubation was carried out for 1 h. This entire procedure was repeated twice. Finally, cells were washed twice, and used for MSLR. As controls, EC were incubated with C′ only, prior to the initiation of MSLR.

Separation of cell subpopulations by immunoglobulin (Ig) coated plates

A modification of the plate technique described by Wysocki & Sato (1978) and Reinherz *et al.* (1981) was utilized to separate cell subpopulations for MSLR. Briefly, plastic plates (Corning 14830, NY, U.S.A.) were individually coated with 10 μg of affinity-purified goat anti-mouse Ig (Tago, Inc, Burlingame, CA, U.S.A.) in 10 ml of PBS and kept at 4°C for 16 h. The plates were then washed three times with 10 ml of cold PBS by a gentle swirling motion, filled with PBS supplemented with 2% FCS and 2% penicillin-streptomycin, and then maintained at room temperature until the addition of MCAB coated cells. This PBS medium was removed prior to addition of the cell suspension.

Concurrently, 5–6 × 10^6 EC were suspended in culture medium complemented with either OKT6 antibody (diluted 1:200) or anti HLA-Dr MCAB (diluted 1:20). Cells were incubated for 1 h at room temperature, during which time they were gently resuspended three times. Cells were next washed in PBS with 2% FCS, and 3 ml of suspension at a concentration of 3–4 × 10^6/ml was applied to a single goat anti-mouse Ig-coated plate. During incubation, the contents of the plate were gently swirled every 30 min. After 60 min, the supernatant was removed (non adherent (NA) fraction). The adherent (A) cells were then removed by repeated scraping with a rubber policeman and washed with PBS medium. The two fractions of OKT6 treated cell suspensions, NA and A, were used in MSLR. NA and A fractions of OKT6 or anti HLA-Dr MCAB treated cells were used for further electron microscopic studies.

As controls, the A and NA fractions were analysed by indirect immunofluorescence (IF) for the percentage of OKT6 positive and HLA-Dr positive cells. These IF analyses of EC suspensions treated with OKT6 MCAB and then separated on Ig coated dishes demonstrated that the A fraction contained 10–12% of OKT6 positive cells, and the NA fraction 1% of OKT6 positive cells. In EC suspensions similarly treated with anti HLA-Dr, the A fraction contained 15–16% HLA-Dr positive cells and the NA less than 1%.

As controls, cells were directly applied to Ig-coated plates without previous incubation with MCAB. IF analysis showed that the NA and A fractions were neither enriched nor depleted in OKT6 or HLA-Dr positive cells: 2–4% of the cells were positive for each of these antigens using this procedure and this percentage was not different from that noted by IF analysis of unfractionated original EC suspensions.

Mixed skin cell lymphocyte culture reaction (MSLR)

MSLR were performed in U-bottomed microtitre plates (Cook Lab., Alexandria, VA, U.S.A.). In each well, 1×10^5 PB cells were co-cultivated with 1×10^5 allogeneic EC (responder: stimulator (R:S) ratio 1:1) in 0·2 ml culture medium, at 37°C in a humidified atmosphere of 5% CO_2. Non-treated cells or cells pretreated with monoclonal antibodies using the techniques described above were studied. Cultures were established in triplicate. As controls, EC alone and PB cells alone were cultured under culture conditions otherwise identical to those of MSLR.

After 5 days of culture, [^{3}H]thymidine (Amersham, U.K.) was added (1 μCi/well) and the cells were harvested 18 h later. Thymidine uptake was evaluated by means of liquid scintillation counting (SL 3000, Intertechnique, France). In the present study, EC were not irradiated or mitomycin treated (Tanaka & Sakai, 1979) before the initiation of the MSLR. In previous controls, identical thymidine uptake was obtained in MSLR using untreated or irradiated EC; preirradiation of EC does not abrogate the responses (Czernielewski *et al.*, 1982), indicating that the cells which proliferate in MSLR are not the EC. Furthermore, EC have been shown not to grow in the culture conditions used for the MSLR (Eisinger *et al.*, 1979). Results were expressed as geometric means of c.p.m. counts of every triplicated MSLR in every set of experiments.

Immuno-electron microscopy of cell suspensions

OKT6 or anti HLA-Dr MCAB were used at the dilution 1:10 for 30 min at 37°C on cells fixed by 3% paraformaldehyde, after cell separation on Ig coated dishes. Both A and NA fractions were incubated with the MCAB previously used for the cell separation. The murine antibodies on the cell surface were revealed by a peroxidase-conjugate (1:10 dilution, 30 min at 37°C, rabbit anti-mouse Ig-peroxidase, Nordic). The peroxidase activity was revealed using 3-3′ diaminobenzidine. Cells were postfixed for 20 min with osmium tetroxide and embedded in epoxy resin. Ultrathin sections were examined without staining with an Hitachi HU12A electron microscope. Some sections were post-stained with lead citrate and uranyl acetate.

As controls, the same reactions were performed using a negative serum (non-immune mouse serum). The non-specific fixation of the conjugate was controlled by using PBS instead of the monoclonal antibodies.

Statistical analysis

Statistical analysis was performed using Student's t-test, with $P < 0{\cdot}05$ indicating significant differences.

RESULTS

MSLR

Allogeneic MSLR with EC pretreated with OKT6 + C′ or anti-HLA-Dr + C′. PB cells and allogeneic EC from ten different donors were used. In each case, the same EC were used either untreated or after treatment with complement alone, OKT6 + C′ or anti HLA-Dr + C′. As compared with results in MSLR using untreated EC, stimulation with OKT6 + C′ treated EC was significantly reduced ($P < 0{\cdot}01$) (Table 1a). PB cell stimulation was abolished when anti HLA-Dr + C′ treated EC were used ($P < 0{\cdot}001$ as compared with control MSLR with untreated cells). Pretreatment of EC by complement alone had no effect (Table 1a).

TABLE 1. Proliferative responses in allogeneic MSLR using (a) PB cells and either untreated EC (L + EC) or EC suspensions pre-incubated with complement alone (L + EC(C′)), with OKT6 and complement (L + EC (OKT6 + C′)), or with anti HLA-Dr monoclonal antibody and complement (L + EC (HLA-Dr + C′)) (ten cases), and (b) PB cells and, as stimulator cells, either OKT6 + EC depleted suspensions (non adherent: NA (OKT6-) EC), OKT6 + EC 'enriched' suspensions (adherent: A (OKT6 +) EC) after panning, or untreated EC suspensions (EC) (six cases). L indicates the proliferation rate of PB cells when cultured in the absence of EC. Results are expressed as geometric means ($\log_{10}$) ± s.d. of c.p.m.

(a)

L	L + EC	L + EC (C′)	L + EC (OKT6 + C′)	L + EC (HLA-Dr + C′)
3·538 ± 0·280	4·680 ± 0·202	4·499 ± 0·128	4·112 ± 0·510	3·616 ± 0·377
	$P<0{\cdot}001$*	$P<0{\cdot}001$*	$P<0{\cdot}01$*	NS*
	└── NS	──┘		
	└──── $P<0{\cdot}01$	────	──┘ └──── $P<0{\cdot}02$	──┘
	└────	──── $P<0{\cdot}001$	────	──┘

(b)

L	L + EC	L + A (OKT6 +) EC	L + NA (OKT6-) EC
3·392 ± 0·114	4·333 ± 0·136	4·366 ± 0·202	3·767 ± 0·172
	$P<0{\cdot}001$*	$P<0{\cdot}001$*	$P<0{\cdot}01$*
	└──── NS	──┘	
	└────	$P<0{\cdot}001$ ────	──┘

* As compared with results with L. NS = Not significant.

Allogeneic MSLR with EC depleted or enriched in OKT6 reactive cells. Six different experiments (with different EC and PB cells in each case) were conducted. As seen in Table 1b, a marked reduction of cell proliferation was obtained when the NA population was used ($P<0{\cdot}001$). However, these OKT6 reactive depleted EC were able to induce a PB cell proliferation that was still higher than their spontaneous proliferation ($P<0{\cdot}01$). Stimulation obtained with the A population did not significantly differ from the proliferation in MSLR conducted with untreated EC.

Immuno-electron microscopic investigations

Immunoperoxidase labelling and EM observations of OKT6 reactive and HLA-Dr positive EC were performed on both the adherent (A) and non-adherent (NA) population obtained after separation of the unfractionated cells on Ig-coated plates (enriched and depleted population respectively).

OKT6 enrichd (A) populations. OKT6 labelled cells could be identified by the presence of an electron-dense granular deposit on their surface (Fig. 1a). All these OKT6-positive cells contained Birbeck granules (Fig. 1 inset) characteristic of Langerhans cells. T6 antigens were expressed exclusively on the cell membrane and staining within the cytoplasmic structures was never observed. Contaminating keratinocytes and melanocytes exhibited no labelling of their surfaces or cytoplasm.

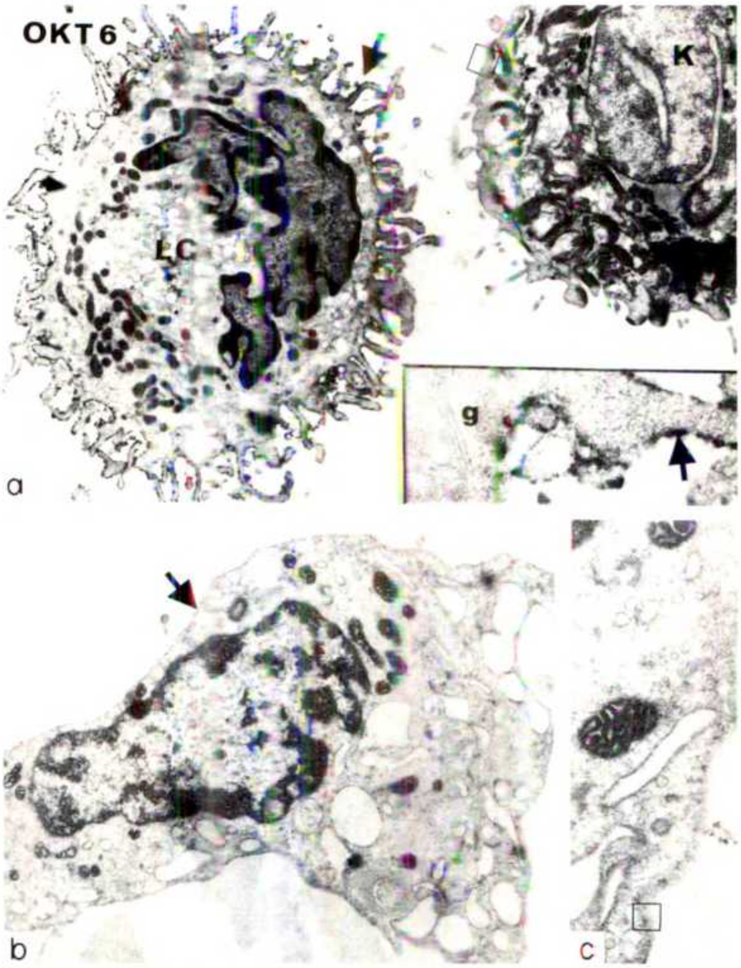

FIGURE 1. (a) Langerhans cells (LC) with Birbeck granules (g). Peroxidase labelling with OKT6 MCAB (arrow); unlabelled keratinocytes (K) (× 5,520; inset × 21,600). (b and c) Indeterminate cell: no labelling with OKT6 MCAB (arrow) (□) (× 5,520; inset × 21,000).

OKT6 depleted (NA) populations. No OKT6 positive cells were observed. Among the negative cells, indeterminate cells, defined by the absence of Birbeck granules, melanosomes and tonofilament bundles and the presence of cytoplasmic vesicles, numerous filaments and dense mitochondria, were observed (Fig. 1b, c). No cytoplasmic digitations were observed on cells recovered from the NA fractions. These indeterminate cells did not express the T6 specificity and were never observed in the OKT6 enriched-adherent (A) cell population.

HLA-Dr enriched (A) populations. Two kinds of HLA-Dr positive cells could be noted. Firstly (Fig. 2a, b, c), typical LC (C1) with numerous villi, Birbeck granules and a positive staining restricted to the plasma membrane, as irregular small spots. Secondly (Fig. 2a, d, e), IC with the morphological characteristics described above and cytoplasmic digitations, the presence of which may be related to the fact that these cells were part of an adherent population.

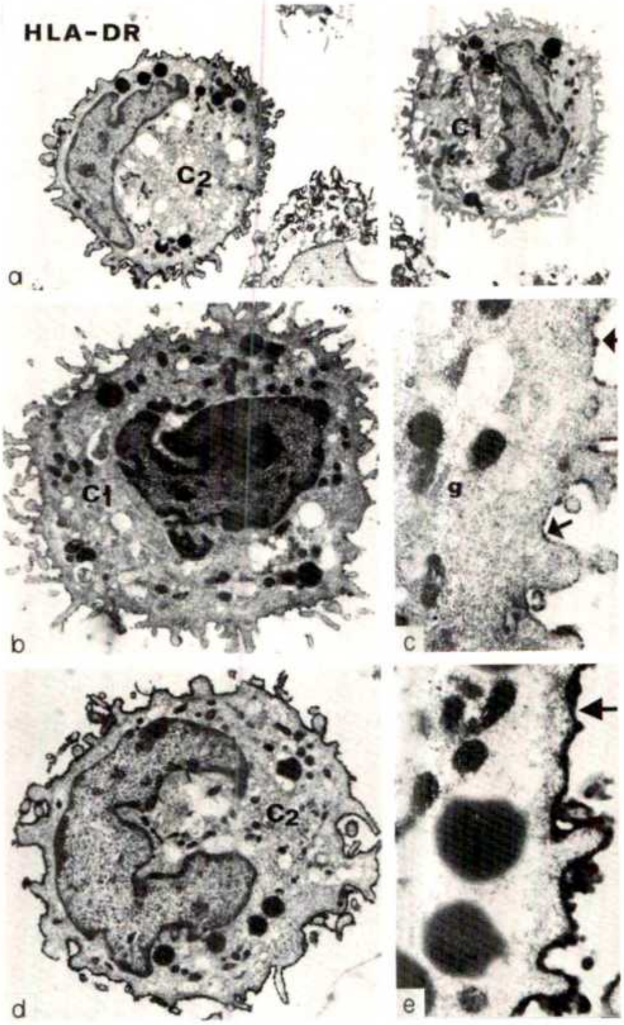

FIGURE 2. Langerhans cells (C1) and indeterminate cell (C2). Peroxidase labelling with HLA-Dr MCAB. (a) × 2,950. (b and c) Higher magnification of Langerhans cells (C1) with Birbeck granules (g) and HLA-Dr staining (arrow): irregular membrane spots (× 4,600; × 22,500). (d and e) Higher magnification of indeterminate cell (C2) with anti-HLA-Dr: intense labelling (arrow) (× 4,600—d; × 22,500—e).

IC were strongly stained by the anti-HLA-Dr MCAB, as a regular dense lineo-granular deposit on the cell membrane. This staining was more intense than that noted on LC membranes. No staining was noted within the cytoplasm. Furthermore, no keratinocyte or melanocyte was HLA-Dr positive.

HLA-Dr depleted (NA) populations. These suspensions were composed only of keratinocytes and some melanocytes. No LC or IC could be observed. All the cells were negative after reaction with anti HLA-Dr MCAB.

Lastly, all controls were negative and confirmed the specificity of the immunolabelling.

DISCUSSION

In MSLR, PB cells proliferate *in vitro* in response to EC. Previous work has shown that this phenomenon may be demonstrated in man and in animals between either allogeneic (Hirschberg & Thorsby, 1975; Lane, Jackson & Ling, 1975; Morhenn *et al.*, 1978) or autologous cells (Beucher & Saurat, 1979; Czernielewski *et al.*, 1982) and that the MSLR is in many ways similar to the one-way mixed lymphocyte reaction (MLR) (Tanaka & Sakai, 1979). In the present study, two different experimental approaches were used to define the epidermal cell population(s) necessary for the MSLR. Firstly, complement mediated lysis using appropriate monoclonal antibodies was used to deplete functionally active cell subsets from EC suspensions. Secondly, these MCAB were utilized to isolate distinct immunoregulatory cell populations by the Ig coated plate technique as described recently (Reinherz *et al.*, 1981).

As shown here and also previously reported with anti HLA-Dr hetero-antiserum (Braathen & Thorsby, 1980; Morhenn *et al.*, 1978), pre-incubation of EC in the presence of anti HLA-Dr serum and complement results in an inhibition of lymphocyte stimulation. Using OKT6 MCAB and complement, we found that such a treatment of EC suspensions leads to a significant reduction of the proliferative response in MSLR. The inhibition was less marked than that noted with anti HLA-Dr MCAB. This may be related to a difference in the relative efficiency of each of these MCAB, or to a difference in the proportion of the cells that express the two specificities. T6 has been shown to represent a surface differentiation antigen specific, among EC, to LC (Fithian *et al.*, 1981; Murphy *et al.*, 1981). LC that represent 2–4% of the cell suspensions used in our MSLR (Eisinger *et al.*, 1979), are known to express the HLA-Dr antigens and to play a major role as antigen-presenting and allo-reactivating cells (Stingl *et al.*, 1980; Breathnach, 1980).

Recently, IC in the epidermis have also been shown to express the HLA-Dr specificity (Rowden *et al.*, 1979). It has also been suggested that keratinocytes, under some pathological conditions (lichen planus, mycosis fungoides, graft-versus-host disease), could express this specificity (Lampert *et al.*, 1981; Mason *et al.*, 1981; Tjernlund, 1981).

The differences in responses in MSLR using either anti HLA-Dr and C′ treated cells or OKT6 and C′ treated cells support the hypothesis that not only LC, but other HLA-Dr positive cells could be involved in lympho-epidermal interactions in MSLR.

The Ig-coated plate technique has already been utilized to isolate PB cell subsets after incubation of unfractionated T-cells with the appropriate antibody specific for T-cell differentiation antigen (Reinherz *et al.*, 1981). We have here adapted this method to obtain both OKT6 and HLA-Dr depleted or enriched EC suspensions, and proceeded with immunomorphological characterization of those various epidermal cell subpopulations.

Our results in electron microscopy indicate two points: among epidermal cells, exclusively Langerhans cells express the T6 antigen, as previously indicated on tissue sections by Murphy *et al.* (1981). Indeterminate cells express the HLA-Dr specificity (Rowden *et al.*, 1979). Moreover, in our study IC appeared T6 negative and the keratinocytes were never found expressing either the T6 or the HLA-Dr specificity. The existence of two distinct kinds of epidermal cells expressing the HLA-Dr antigens (LC and IC) may well explain the different degrees of reduction in MSLR conducted with either OKT6 and C′ treated or anti-HLA-Dr and C′ treated stimulator cells. Furthermore, the reduction but not the abolition in the stimulating ability of EC after depletion in OKT6 cells (using the Ig-coated plate technique) is well related to the presence, in the non-adherent population used, of IC but not of LC, as demonstrated by our electron-microscopic examinations.

A recent report by Chu *et al.* (1982) suggested the presence of T6 antigen on epidermal cells lacking Birbeck granules. The difference between this and our present findings may be explained by different methodological approaches used, as our electron-microscopic observations were made after a prior enrichment or depletion of EC suspensions.

The present data indicate that two dendritic epidermal cells may be defined by specific markers: the LC with Birbeck granules that express T6 and HLA-Dr antigens (T6 + Dr + granules +) and the IC, without Birbeck granules and T6 antigen, but expressing the HLA-Dr specificity (T6 − Dr + + granules −). Both are necessary for an optimal *in vitro* stimulation of peripheral blood lymphocytes by epidermal cells. Further functional and ultrastructural studies should be undertaken to determine more intimately the exact relationship between those two kinds of cell and the cells from the mono-histiocyte lineage.

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Human epidermal Langerhans cells are more potent than blood monocytes in inducing some antigen-specific T-cell responses

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SUMMARY

The abilities of human epidermal Langerhans cells and peripheral blood macrophages to induce specific T-lymphocyte responses to purified protein derivative (PPD), herpes simplex virus and nickel sulphate were compared. On a cell to cell basis the Langerhans cells were found to be about as potent as adherent cells in inducing T-cell responses to PPD, more potent in inducing responses to herpes simplex virus, and several times more potent in inducing responses to nickel. These data indicate that human epidermal Langerhans cells are highly specialized in antigen presentation, particularly for nickel sulphate. They may play an important role in inducing contact sensitivity dermatitis.

There is now considerable evidence that human epidermal Langerhans cells play an important role in the immune responses in the skin. In recent publications we have reported that: (i) human epidermal Langerhans cells can stimulate allogeneic T lymphocytes to proliferate *in vitro* (Braathen & Thorsby, 1980a); (ii) they can substitute for macrophages in inducing antigen-specific proliferative T-cell responses to purified protein derivative of tuberculin (PPD), herpes simplex virus antigen (HSV-Ag) and live virus (HSV) *in vitro* (Braathen & Thorsby 1980a; Braathen *et al.*, 1980; Braathen & Thorsby, 1980b); (iii) they are capable of inducing a strong proliferative T-cell response to the contact allergen nickel sulphate (Braathen, 1980); (iv) the Langerhans cell-dependent antigen responses are restricted by the HLA-D/DR molecules, i.e a proliferative response *in vitro* to PPD and HSV-Ag, similar in strength to that seen in autologous combinations, can be obtained only when the epidermal cells share both HLA-D/DR determinants with the sensitized T-cell donor (Braathen & Thorsby, 1982).

We have now compared the potency of epidermal Langerhans cells and peripheral blood adherent cells in inducing a T-cell response to PPD, HSV-Ag and nickel sulphate.

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MATERIALS AND METHODS

Cell donors

Cells were obtained from tuberculin positive individuals and/or individuals having recurrent herpes labialis, and from individuals with contact allergic dermatitis to nickel. Sensitization to HSV was confirmed by serological tests as previously described (Braathen *et al.*, 1980) and sensitization to nickel was performed according to the European Standard series with 5% nickel sulphate in petrolatum.

PPD of tuberculin

PPD solution in phosphate buffer without preservative (Veterinary Institute, University of Oslo) was used, freshly diluted in medium RPMI 1640 supplemented with penicillin and streptomycin.

Herpes simplex virus antigen

Lyophilized herpes simplex type 1 CF (complement fixation) antigen produced in monkey kidney cells (Orion Diagnostica, Helsinki) was used, freshly diluted in medium RPMI 1640 supplemented with penicillin and streptomycin.

Nickel sulphate solution

Nickel II sulphate ($NiSo_4 \times 6H_2O$) 'zur Analyse' with a mol. wt of 262.86 was obtained from Merck AG, Darmstadt, W. Germany (Art. no. 6727). A stock solution of 1% nickel sulphate in 0·9% saline was prepared, and additional dilutions were made with medium RPMI 1640. Usually three dilutions were used in experiments: 1:25, 1:50 and 1:100, but in some experiments higher dilutions were also used.

Epidermal cells

Epidermal cell suspensions were made by trypsinization of epidermis sheets obtained with the suction blister technique, as previously described (Braathen & Thorsby, 1980a). The cells were suspended in medium RPMI 1640 with L-glutamine (Gibco-Bio-Cult, Glasgow) supplemented with penicillin, streptomycin and 20% normal human serum. Viability determined by trypan blue exclusion ranged from 75% to 95%, but was usually more than 90%. The cell suspensions contained 3%–6% Langerhans cells, as judged by immunofluorescence staining of the cell suspensions with a rabbit anti-DR antiserum as described earlier (Braathen & Thorsby, 1980a). No peroxidase positive monocytes/macrophages were detected in the epidermal cell suspensions.

Peripheral blood mononuclear cells

These were separated from defibrinated blood by means of flotation on Lymphoprep (Nyegaard & Co., Oslo, Norway).

T lymphocytes

T lymphocytes were prepared from the isolated mononuclear cells by rosetting with AET-treated sheep red cells as described earlier (Braathen & Thorsby, 1980a).

Adherent cells/macrophages

Macrophages (M0) were prepared from peripheral blood mononuclear cells by means of the adherence to tissue culture flasks as described earlier (Braathen & Thorsby, 1980a).

Cell culture techniques

The cells were incubated in round-bottomed microtitre plates (Model 15-MCR 96, Linbro Scientific Inc., Hamden, Conn. U.S.A.) in a humid 5% CO_2 atmosphere at 37°C for 5 days. T cells (5×10^4) were co-cultured with 5×10^3 autologous adherent cells, or with 5×10^4 or 1×10^5 autologous epidermal cells, with and without antigen added. In some experiments peripheral blood mononuclear cells adjusted to 5×10^4 T cells were also cultured with and without antigen. Each well contained 170 μl medium RPMI 1640 supplemented with antibiotics and 20% normal human serum. [^{3}H]thymidine was added 18 h before harvesting, which was performed with a semi-automatic multiple cell culture harvester (Skatron, Lierbyen, Norway). Incorporation was measured as mean c.p.m. of triplicate cultures + s.e.

The statistical method used was the Wilcoxon matched pair signed-ranks test.

RESULTS

PPD-specific T-cell responses using macrophages and epidermal cells

In Table 1 the results of ten experiments are shown using 5×10^3 Mø, 5×10^4 or 10^5 epidermal cells (Ec) containing approximately $2{\cdot}5 \times 10^3$ or 5×10^3 HLA-DR positive Langerhans cells in the cultures. The responses using Mø or Ec were quite similar: in five of the experiments the highest responses were obtained with Mø and in the other five experiments the highest responses were obtained with Ec. The mean PPD T-cell response obtained with 5×10^3 Mø was of the same order of magnitude as the mean response obtained with the same number (5×10^3) of Langerhans cells. These results indicate that peripheral blood adherent cells and epidermal Langerhans cells are equally potent in inducing PPD specific T-cell responses.

HSV-specific T-cell responses using macrophages and epidermal cells

Figure 1 shows a typical experiment with varying numbers of macrophages and epidermal cells.

TABLE 1. Ec and Mø-dependent T-cell responses to PPD

	T-cell responses to PPD*			
		Mø × 10^{-3}	Ec × 10^{-4}	
Exp. no.		(5)	5	10
1	254 ± 91†	3868 ± 440	4250 ± 1062	3926 ± 840
2	295 ± 96	2611 ± 613	6047 ± 593	6525 ± 702
3	169 ± 47	13,239 ± 1317	7949 ± 569	5398 ± 388
4	69 ± 23	7720 ± 349	3157 ± 263	—
5	4445 ± 1302	25,406 ± 1413	39,501 ± 958	39,586 ± 1713
6	644 ± 330	7878 ± 1296	8648 ± 802	12,353 ± 918
7	201 ± 64	2233 ± 220	1669 ± 486	—
8	2571 ± 458	17,558 ± 1516	14,573 ± 148	16,489 ± 1271
9	826 ± 291	32,094 ± 1705	13,252 ± 441	—
10	4856 ± 527	13,057 ± 962	44,733 ± 3099	39,206 ± 354
Mean	1433	12,566	14,378	17,640

* T cells (5×10^4).

† Mean c.p.m. ± s.e. of triplicate cultures.

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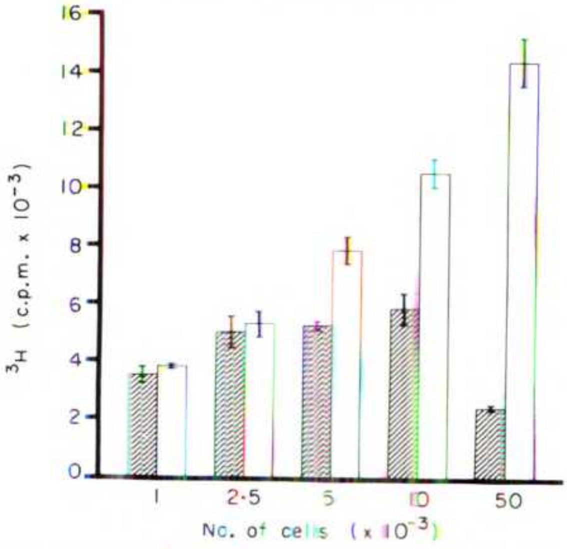

FIGURE 1. Ec (□) and Mø-dependent (▨) T-cell responses to HSV-Ag. T cells (5×10^4). Values are in mean c.p.m. + s.e.

An increasing number of epidermal cells in the cultures increased the T-cell response to HSV-Ag, while the T-cell response obtained with Mø reached a maximum with $5–10 \times 10^3$ Mø in the cultures.

In Table 2 the results of eight experiments are shown using 5×10^3 Mø or 5×10^4 or 10^5 epidermal cells containing approximately $2{\cdot}5 \times 10^3$ or 5×10^3 HLA-DR positive Langerhans cells. In one experiment the response obtained with Mø was similar to the response obtained with 10^5 Ec, while in the other seven experiments the responses obtained with Ec were higher ($P < 0{\cdot}02$). The mean values show that a two or three times stronger HSV-Ag T-cell response

TABLE 2. Ec and Mø-dependent T-cell responses to HSV-Ag

	T-cell response to HSV-Ag*			
			Ec × 10^{-4}	
Exp. no.		Mø × 10^{-3} (5)	5	10
1	†171 ± 76	3393 ± 100	1829 ± 176	3041 ± 680
2	293 ± 154	5236 ± 113	14,407 ± 780	13,822 ± 272
3	527 ± 23	6343 ± 1368	13,336 ± 831	17,986 ± 1398
4	166 ± 84	776 ± 353	3238 ± 152	—
5	400 ± 94	4348 ± 209	9684 ± 1410	—
6	4987 ± 422	12,793 ± 1054	24,252 ± 605	20,228 ± 693
7	383 ± 181	4480 ± 413	10,709 ± 1117	12,267 ± 203
8	303 ± 40	2562 ± 432	3994 ± 149	5035 ± 286
Mean	904	5054	10,181	12,063

* T cells (5×10^4).

† Mean c.p.m. ± s.e. of triplicate cultures.

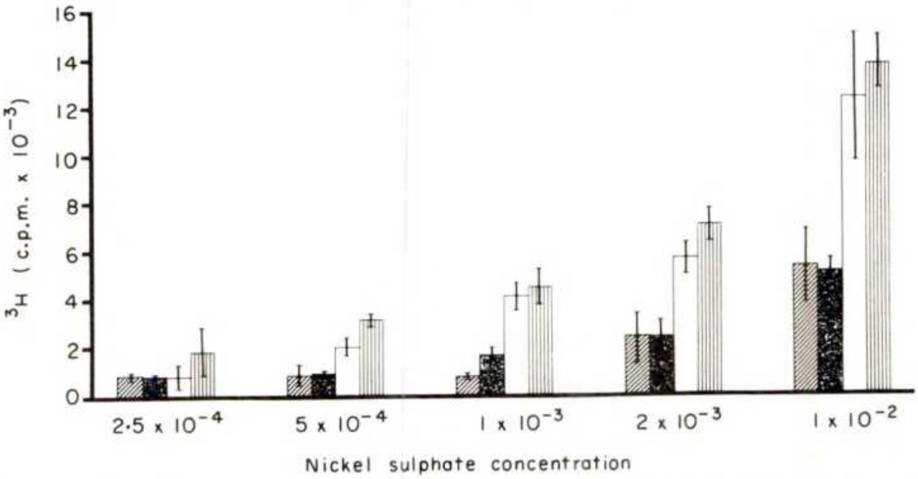

FIGURE 2. Ec and Mø-dependent T-cell responses and peripheral blood mononuclear cell responses to various concentrations of nickel sulphate. T cells (5×10^4). Peripheral blood mononuclear cells (Ly) adjusted to 5×10^4 T cells. Values are in mean c.p.m. + s.e. (▨) Mø (5×10^3); (■) Ly (adjusted to 5×10^4 T cells); (□) Ec (5×10^4); (▥) Ec (1×10^5).

was obtained with Langerhans cells than with the same number of macrophages, which is in contrast to what we found in the PPD response using similar numbers of Mø and Ec. This indicates that the Langerhans cells are more potent in inducing HSV-specific T-cell responses than macrophages in patients with recurrent herpes labialis.

Nickel sulphate-specific T-cell responses using macrophages and epidermal cells

We first investigated the influence of different concentrations of nickel sulphate. Figure 2 shows an experiment comparing the responses obtained with peripheral blood mononuclear cells adjusted to 5×10^4 T cells, and adding to these cells antigen, or antigen plus 5×10^3 Mø, or 5×10^4 or 10^5 epidermal cells. It can be seen that adding Ec always resulted in a more potent response than adding Mø, at all concentrations of nickel sulphate investigated.

Figure 3 shows two typical experiments with purified T cells and antigen plus varying numbers of Ec and Mø in the cultures.

Table 3 shows the overall results of nine experiments using 5×10^3 Mø, 5×10^4 or 10^5 Ec, and a nickel sulphate dilution of 2×10^{-4} added to the wells. In all of the experiments the Ec-dependent responses were the highest ($P < 0{\cdot}01$) and in five of the experiments no significant responses were obtained with macrophages. While the mean Mø-dependent nickel sulphate T-cell response was weak, the response obtained with Ec was approximately six times higher, indicating that the Ec were much more potent inducers of T-cell responses to nickel sulphate in patients with contact allergic eczema.

DISCUSSION

We have shown that human epidermal Langerhans cells are as potent as peripheral blood adherent cells in inducing PPD-specific T-cell responses *in vitro*, about twice as potent in inducing HSV responses and about six times more potent than adherent cells in inducing nickel sulphate-specific responses of T cells from sensitized individuals.

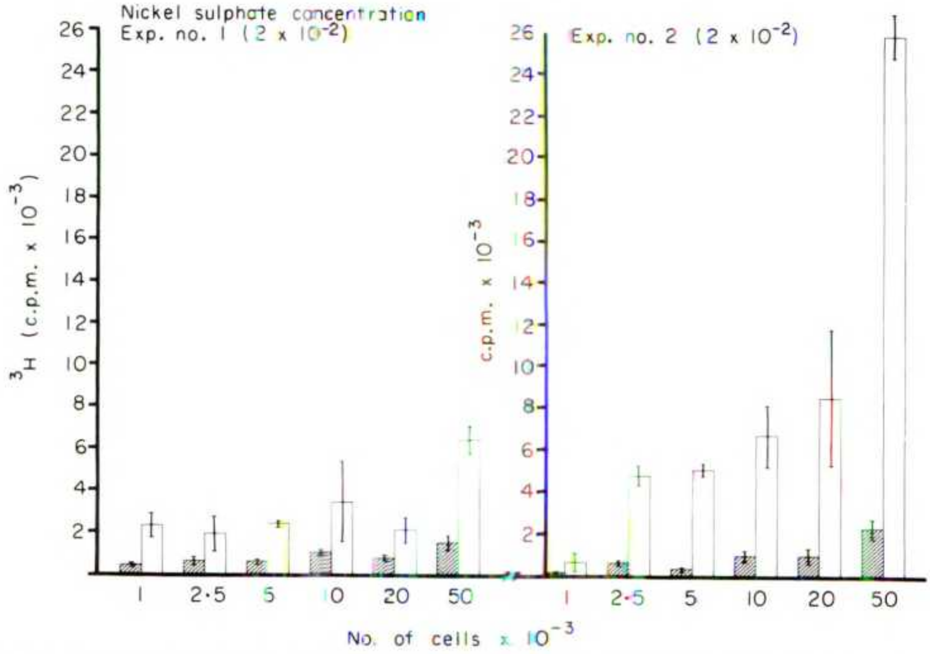

FIGURE 3. Ec and Mø-dependent T-cell responses to nickel sulphate. T cells (5×10^4). Values are in mean c.p.m. ± s.e.

TABLE 3. Ec and Mø-dependent T-cell responses to nickel sulphate

	T-cell response to nickel sulphate*			
		Mø × 10⁻³	Ec × 10⁻⁴	
Exp. no.		(5)	5	10
1	†453 ± 59	5318 ± 1563	12,299 ± 2592	13,681 ± 972
2	84 ± 25	325 ± 9	2601 ± 652	4256 ± 49
3	159 ± 89	210 ± 82	1202 ± 159	1198 ± 107
4	313 ± 134	252 ± 58	5478 ± 823	6689 ± 589
5	273 ± 78	862 ± 233	5082 ± 299	5701 ± 119
6	237 ± 39	263 ± 53	5648 ± 449	—
7	163 ± 16	310 ± 7	13,357 ± 489	—
8	773 ± 165	1313 ± 332	8065 ± 857	—
9	300 ± 103	3530 ± 672	7660 ± 372	—
Mean	306	1376	6821	6305

* T cells (5×10^4).

† Mean c.p.m. ± s.e. of triplicate cultures.

There may be several explanations for this difference in accessory cell function between macrophages and epidermal cells. Firstly, since the epidermal cell suspensions used contained more cells than the macrophage suspensions, one might speculate that HLA-DR negative keratinocytes might also participate as antigen-presenting cells or release Interleukin I, or both, thus resulting in stronger antigen-specific responses using Ec. Since we have previously found that removing the HLA-DR positive cells from Ec abolishes the antigen-presenting capacity of Ec (Braathen & Thorsby, 1980, Braathen *et al.*, 1980), a contribution by HLA-DR negative keratinocytes in antigen presentation is unlikely. However, epidermal cells other than the Langerhans cells may release Interleukin I (Luger *et al.*, 1981). To study this possibility we are currently trying to enrich for HLA-DR positive Langerhans cells and use these as antigen-presenting cells. If Interleukin I release by keratinocytes is the explanation, it is difficult to understand the differences using PPD, HSV and nickel sulphate as antigens.

The second possibility is that different antigens may be handled differently by peripheral blood monocytes/macrophages and Langerhans cells. This needs further studies, again requiring purer populations of Langerhans cells.

Our findings may in some way be related to the type of disease caused by these antigens. Recurrent herpes simplex is accompanied by an extensive immune response in the epidermis. Similarly, nickel contact allergic dermatitis is caused by hypersensitivity to the allergen in the skin without known involvement of other tissue.

Contact hypersensitivity reactions, like delayed-type hypersensitivity reactions seem to occur in two forms, one evanescent and the other long-lived. It has been shown that both the anatomical site where antigen is first encountered by the immune system and the nature of the cells which present the antigen, determine whether a contact sensitivity reaction will ensue, and also whether it will be evanescent or long-lasting (Ptak *et al.*, 1980). Studies in animals have implied that Langerhans cells can effectively present antigen, where other antigen-presenting cells may cause suppression, which might suggest that Langerhans cells are able to activate effector T cells that are relatively resistant to suppression, or alternatively that the distinctive feature of these antigen presenting cells is their inability to interact with the cells of the suppressor circuit (Ptak *et al.*, 1980). Further studies on the Langerhans cells may lead to a better understanding of many skin diseases in which immune responses are involved.

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An immunoelectron microscopy study of the relationship between herpes gestationis and polymorphic eruption of pregnancy

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SUMMARY

Five patients with typical bullous herpes gestationis and five patients with polymorphic eruption of pregnancy were studied. Shave biopsies were taken from both involved and uninvolved skin and examined by routine immunofluorescence. They were also examined by immunoelectron microscopy employing a multistep peroxidase–antiperoxidase technique which has been shown to be more sensitive than immunofluorescence. Despite the increased sensitivity of immunoelectron microscopy all cases of polymorphic eruption of pregnancy were completely negative. Although there is a considerable clinical and histopathological overlap between herpes gestationis and polymorphic eruption of pregnancy our findings indicate that these conditions are pathogenetically distinct and should be classified as separate disorders.

Polymorphic eruption of pregnancy (PEP) is a pruritic urticarial eruption which develops in and around the abdominal striae towards the end of pregnancy. It is known by several synonyms including toxaemic rash of pregnancy (Bourne, 1962), late onset prurigo of pregnancy (Nurse, 1968), pruritic urticarial papules and plaques of pregnancy (Lawley *et al.*, 1979) and toxic erythema of pregnancy (Holmes *et al.*, 1982). Recently we proposed the name 'polymorphic eruption of pregnancy' as the lesions consist not only of papules and plaques but also of target lesions, polycyclic weals and vesicles (Holmes & Black, 1982). The separation of PEP from herpes gestationis (HG) is supported by immunofluorescence (IMF) studies which demonstrate C3 deposition at the basement membrane zone (BMZ) in all cases of HG, yet are consistently negative in PEP (Holmes *et al.*, 1982). However, there is undoubtedly a considerable clinical and

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histopathological overlap between PEP and the pre-bullous stage of HG and it has been suggested that PEP is, in fact, a mild form of HG (Nurse, 1968). Also Holubar, Konrad & Stingl (1977) have shown that in HG small deposits of immunoreactants may escape detection by IMF and yet be demonstrable by immunoelectron microscopy (IEM). This finding brought into question the reliability of negative IMF in PEP and necessitated a re-evaluation of the relationship between PEP and HG by the use of IEM.

METHODS

Five patients with typical bullous HG and five with PEP were studied. We have recently described our diagnostic criteria for PEP (Holmes *et al.*, 1982). Shave biopsies were taken from lesional or normal-appearing skin. At the time of biopsy three cases of PEP and four of HG had active disease (Table 1). The shave biopsies were bisected; one-half was frozen in liquid nitrogen for IMF studies and the other half was placed in phosphate-buffered saline (PBS) for IEM.

IMF studies were performed using standard direct IMF techniques (Beutner & Nisengard, 1973). Commercially obtained fluorescein-labelled rabbit anti-human IgG, IgM, IgA, C3 and fibrinogen (DAKO) were used (Holmes *et al.*, 1982).

Immunoelectron microscopy was performed using a peroxidase anti-peroxidase technique similar to that described by Holubar *et al.* (1975). The shave biopsy was cut into thin strips measuring less than 0·5 mm in thickness and rinsed for 30 min in PBS. This was followed by incubation in normal swine serum, diluted 1 : 10 with PBS, for 15 min.

Without being rinsed, the specimens were transferred to the following sequence of antibodies to demonstrate IgG deposition. Rabbit anti-human IgG, swine anti-rabbit immunoglobulin and horseradish peroxidase rabbit anti-horseradish peroxidase complex. A similar sequence employing rabbit anti-C3 as the primary antibody was used to demonstrate C3 deposition. Commercially available antibodies diluted with PBS were used for all incubation steps. (DAKO immunoglobulins: rabbit Ig to human C3 complement A062; rabbit Ig to human IgG A090; swine Ig to rabbit immunoglobulins Z196; soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase Z113.) All steps were carried out at room temperature with incubations of 30 min for each step and intermediate rinsing in PBS. After the final rinse in PBS the specimens were incubated for 30 min in 0·05% diaminobenzidine (3, 4, 3′, 4′-tetra-amino-phenyl hydrochloride) diluted in PBS. They were fixed in 2% glutaraldehyde, post-fixed in osmium tetroxide and embedded in Araldite. Ultrathin sections were cut with a LKB ultratome III, stained with uranylacetate and/or lead citrate and examined in a Philips 300 electronmicroscope.

Controls were performed by omission of one step of the incubation sequence.

RESULTS

Immunofluorescence

In all patients with HG, direct IMF demonstrated linear deposition of C3 at the BMZ and in one patient there was also deposition of IgG. No fluorescence was observed after incubation with fluorescein-labelled anti-IgA, anti-IgM or anti-fibrinogen. In PEP the IMF findings were entirely negative.

Immunoelectron microscopy

In HG, IEM demonstrated C3 deposition at the BMZ in all patients (Fig. 1) and IgG deposition in four out of five cases. The electron dense reaction product was deposited in a band-like

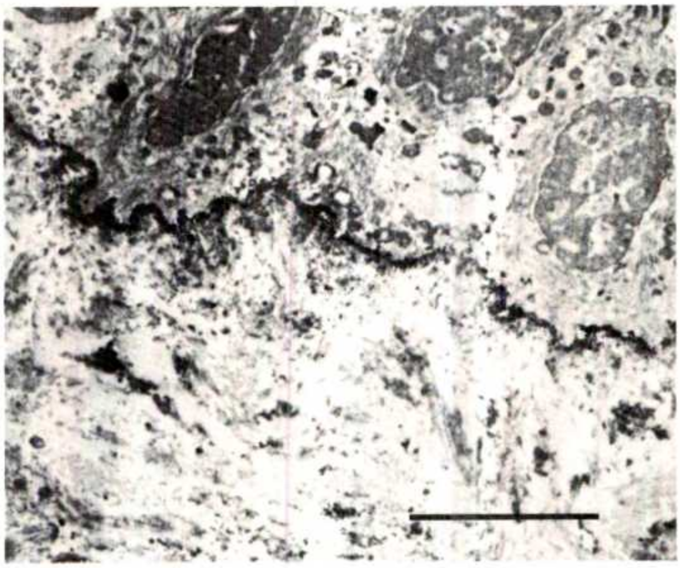

FIGURE 1. Herpes gestationis: immunoelectron microscopy demonstrating C3 deposition at the BMZ. (Scale = 5 μm.)

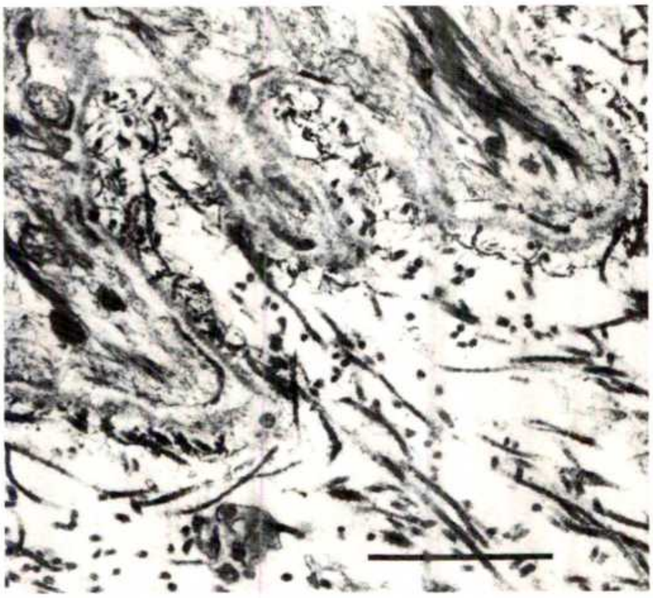

FIGURE 2. Polymorphic eruption of pregnancy: negative immunoelectron microscopy. (Scale = 1 μm.)

TABLE 1. Immunopathological findings in HG and PEP

Patient	Diagnosis	Gestation	Clinical appearance of biopsy	Disease activity	Direct IMF		Direct IEM	
					C3	IgG	C3	IgG
J.L.	HG	36 Weeks	Normal	Active	+	–	+	+
P.G.	HG	10 Months p.p.*	Lesional	Active	+	+	+	+
T.G.	HG	22 Months p.p.	Normal	Active	–	–	+	+
J.B.	HG	7 Years p.p.	Normal	Inactive	+	–	+	+
C.W.	HG	7 Years p.p.	Normal	Inactive	–	–	+	–
S.R.	PEP	30 Weeks	Lesional	Active	–	–	–	–
C.E.	PEP	39 Weeks	Lesional	Active	–	–	–	–
F.A.	PEP	1 Week p.p.	Normal	Inactive	–	–	–	–
A.B.	PEP	6 Weeks pp.	Lesional	Active	–	–	–	–
B.H.	PEP	5 Months p.p.	Normal	Inactive	–	–	–	–

* p.p., *Post partum.*

pattern along the BMZ, most of it obscuring the lamina lucida. In some areas the granular deposits were found reaching into the papillary dermis, but no reaction product was observed in the intercellular spaces between the basal epidermal cells. The IEM findings in PEP were completely negative (Fig. 2). The results are summarized in Table 1.

DISCUSSION

When pruritic urticarial eruptions develop during pregnancy there is often considerable difficulty distinguishing between HG and PEP. Both disorders usually begin on the abdomen and can become widespread with urticarial papules and plaques, polycyclic erythematous wheals, target lesions and vesicles (Holmes *et al.*, 1982). Their histopathology is also similar with epidermal and upper dermal oedema, vesicular spongiosis and a perivascular lymphohistiocytic infiltrate with variable numbers of eosinophils (Holmes *et al.*, 1982). In view of their clinical and histopathological overlap it is possible that PEP may in fact be a mild form of HG and that IMF is not sensitive enough to detect the deposits of C3 in PEP. Therefore, we investigated this possibility employing a peroxidase–antiperoxidase IEM technique which Holubar *et al.* (1977) have shown to be more sensitive than IMF.

All of our cases of HG were IMF proven with C3 deposition at the BMZ. Our IEM studies confirmed the deposition of C3 and also they revealed deposits of IgG at the BMZ in four out of five patients. In the only patient with HG who had negative IEM for IgG the biopsy was taken 7 years *post partum* at which time the skin disease had been inactive for 4 years. Therefore, we suggest that all active cases of HG would demonstrate IgG deposition at the BMZ if studied by IEM.

Although we confirmed that IEM was superior to IMF in the detection of immunoreactant deposits, this technique revealed no evidence of C3 or IgG at the BMZ in PEP. Thus we conclude that HG and PEP are immunopathologically distinct and that they should be classified as separate disorders.

In clinical practice the examination of lesional skin by direct IMF for C3 remains the most convenient and reliable procedure for the differentiation of HG and PEP.

ACKNOWLEDGMENT

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Papular polymorphic light eruption: an immunoperoxidase study using monoclonal antibodies

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SUMMARY

Biopsy specimens of papules taken from eight patients with polymorphic light eruption were examined by immunoperoxidase techniques employing monoclonal antibodies. In each case, most infiltrating mononuclear cells were T cells. The majority of T cells were T8-positive (cytotoxic/suppressor) in four cases and T4-positive (helper/inducer) in two. In two cases, approximately equal numbers of both T cell subsets were present. In only three cases were rare B cells identified by their reactivity with anti-IgM antibody. M1-positive mononuclear cells (macrophages) represented less than 5% of cells infiltrating the dermis. In five subjects, anti-T6 antibody stained increased numbers of dermal mononuclear cells considered to be Langerhans/indeterminate cells. The pathogenesis of papular polymorphic light eruption may involve injury to upper dermal venules mediated by T cells and Langerhans/indeterminate cells.

Several observations suggest that cellular immunity is important in the pathogenesis of polymorphic light eruption (PMLE). Lesions appear 2 h to 5 days after sunlight exposure and may recur in non-exposed but previously affected sites (Morison, Parrish & Epstein, 1979). The constant and characteristic histological feature is a densely aggregated perivascular infiltrate of mononuclear cells (Wright & Winer, 1960; Epstein, 1980). To characterize the infiltrating mononuclear cells in lesions of PMLE, well-developed papules and sun-exposed clinically normal adjacent skin from subjects with PMLE were studied with monoclonal antibodies which react with various cell subsets.

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METHODS

Subjects

Five male and three female subjects (aged 25–49 years) with a non-scarring, predominantly papular photoeruption induced 1–48 h after exposure to sunlight were selected prospectively from the Light and Pigment Clinic of the Massachusetts General Hospital (Table 1). The duration of the photosensitivity varied from 2 to 25 years. Eczematous patches were not present and there was no history of photosensitive atopic dermatitis in any subject. Photocontact dermatitis and use of systemic photosensitizers were excluded by history. Systemic symptoms of lupus erythematosus were absent. Each subject had a normal complete blood count and erythrocyte sedimentation rate and negative or low (< 1 : 32) antinuclear antibody titre. Direct immunofluorescence study of lesional skin showed a negative lupus band test in all cases (Muhlbauer *et al.*, 1982a). Subject 3, who had papular PMLE for 25 years and solar urticaria for 3 years, developed weals after indoor phototesting to either long-wave ultraviolet or visible radiation.

Skin specimens

Application of topical sunscreens was the only therapy used for 4 weeks before biopsy. Xylocaine 1%, with or without adrenaline, was injected around a well-developed single papule (induced by natural sunlight) on the upper or lower extremity, from which a 4 mm punch biopsy

TABLE 1. Nature of inflammatory infiltrate in papular polymorphic light eruption

	Percentage of cells staining in dermis*						
Patient number	M‡	B§	T6	Ia¶	T4 + T8	T3	T4/T8 ratio†
1	Rare	0	15	70	80	65	1 : 1
2	5	Rare	5	60	80	70	7 : 1
3: Papule	Rare	0	30	70	85	80	3 : 4
Weal	ND	0	30	70	55	ND	1 : 2
4	5	0	5	60	65	80	1 : 1
5	5	0	15	60	75	ND	1 : 2
6	5	0	10	60	85	85	3 : 4
7	5	Rare	Rare	40	90	+ +	3 : 2
8	5	Rare	10	60	90	80	4 : 5

ND = not done.

* Percentages given represent rough estimates of the number of stained mononuclear cells in perivascular aggregates compared with the total number of infiltrating mononuclear cells present. Accurate enumeration of stained cells in cellular aggregates is not possible.

† Calculated by dividing counts of T4-positive cells by the counts of T8-positive cells from perivascular areas in at least five corresponding high power fields on adjacent sections.

‡ Monocytes/macrophages were identified as mononuclear cells stained by anti-M1 antibody.

§ B cells were detected by a monoclonal antibody directed to IgM.

¶ Ia-like antigens were detected by the use of anti-I2 antibody.

+ + = Staining too faint for assessment.

specimen was obtained. As determined from the subject's history, the age of the lesions ranged from 1 to 7 days. Subjects 1, 2 and 3 also underwent a 3 mm punch biopsy of sun-exposed clinically normal skin 1–3 cm away from the papule. For controls, a single 4 mm biopsy specimen of normal skin taken from an extensor forearm was obtained from two women and one man aged 20–34. In subject 3, a weal was biopsied 30 min after exposure to 4·2 J/cm^2 of long wave ultraviolet radiation from a xenon arc source (n order to study solar urticaria. Biopsy specimens of normal skin of control subjects and adjacent exposed skin from subjects 1 and 2 were frozen in OCT Compound (Ames Co., Div. of Miles Laboratory, Elkhard, IN) and stored at −70°C. All specimens taken from papules and all specimens from subject 3 were immediately bisected. Half was placed in 10% buffered formalin for routine histology, and the other half was frozen in OCT Compound and stored at −70°C.

Monoclonal antibodies

A series of monoclonal antibodies reactive with lymphocyte subsets, monocytes and Langerhans cells were used. Anti-T3 antibody reacts with 100% of peripheral E-rosette positive T cells (Reinherz & Schlossman, 1980). Anti-T6 antibody, which reacts with a majority of cortical thymocytes but not with peripheral T cells (Bhan *et al.*, 1980), has recently been shown to react with Langerhans/indeterminate cells in the skin (Poppema *et al.*, 1981; Murphy *et al.*, 1981; Fithian *et al.*, 1981; Murphy *et al.*, 1982). Anti-T4 and anti-T4B (clone SFCI-12T4D11, provided by Dr Ellis Reinherz, Sidney Farber Cancer Institute, Boston, MA; Reinherz *et al.*, 1982) antibodies react with T-cell antigens associated with helper/inducer function. Tissue section staining with anti-T4B is similar to, although more intense than, that of anti-T4 antibody (Bhan *et al.*, 1981a). Anti-T8 antibody reacts with an antigen on T cells associated with cytotoxic/suppressor function (Reinherz & Schlossman, 1980). Anti-I2 antibody reacts with the non-polymorphic regions of human Ia-like (HLA-DR) antigens and labels the vast majority of B cells and monocytes, 20% of null cells, 30–40% of activated T cells, and virtually no resting T cells (Nadler *et al.*, 1981). Monoclonal anti-M1 antibody, reactive with monocytes, natural killer cells and granulocytes, was employed (Breard *et al.*, 1980). A monoclonal anti-IgM antibody reactive with B cells was also used (Bhan *et al.*, 1981b).

Immunoperoxidase procedure

Frozen sections 4 μm thick were air-dried, fixed in acetone for 5 min and stained by a four-step peroxidase-anti-peroxidase (PAP) method (Bhan *et al.*, 1980; Poppema *et al.*, 1981). Sections were first incubated with a 1:100 to 1:500 dilution of mouse ascites containing either one monoclonal antibody, or a mixture of monoclonal anti-T4B and anti-T8 antibodies, for 60 min at room temperature followed by 30-min incubations with rabbit anti-mouse immunoglobulin (Ig) (Dako Immunoglobulins, Copenhagen, Denmark), swine anti-rabbit Ig, and lastly with peroxidase-rabbit-antiperoxidase reagent (Dako Immunoglobulins). Between each incubation, sections were washed in three changes of PBS. In some instances staining was confirmed with an avidin-biotin technique (Hsu, Raine & Fanger, 1981) employing mouse monoclonal antibody as the first step, followed by incubations with biotin-labelled horse anti-mouse Ig and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA).

Staining was achieved by incubation of sections in an acetate buffer solution (pH 5·0) which contained 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI), dimethylformamide and hydrogen peroxide. The sections were washed in acetate buffer and mounted in Elvanol. Some sections were counterstained with Gill's triple strength haematoxylin.

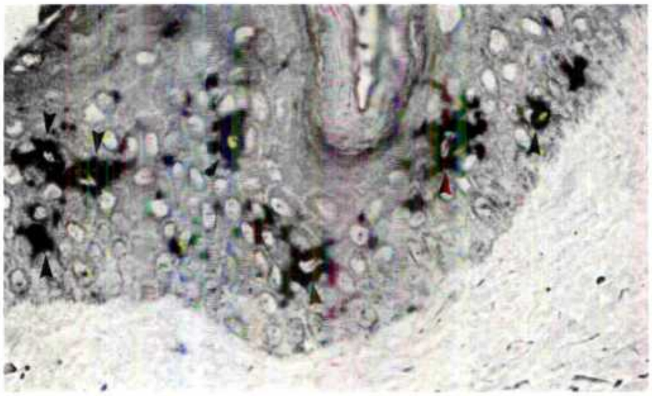

FIGURE 1. Exposed skin adjacent to a papule of PMLE (Subject 2) showing T6-positive dendritic cells in the epidermis (arrows) (× 400). Nuclei appear as central oval zones of non-reactivity.

Counting methods

Sections from each specimen were stained by the PAP technique using either anti-I2 or anti-T6 antibody. Intraepidermal dendritic cells were counted only if the nucleus, visible as negative staining, was present (Fig. 1). The length of epidermis for each section was measured with an ocular micrometer (Zeiss W KPL, 10X; accuracy < 10 μm) in a Zeiss light microscope. The number of cells stained by each antibody was divided by the total epidermal length surveyed from all sections (two or three) on the slide to obtain the number of positive cells per linear millimetre.

The number of T4-positive and T8-positive cells were counted within the perivascular areas of at least five corresponding high power fields on adjacent sections (< 50 μm apart) stained with either anti-T4B or anti-T8 antibodies, and the ratio of T4-positive to T8-positive cells was calculated.

Statistical methods

Student's t-test was employed.

RESULTS

Routine light microscopy

Papules. The epidermis of all papules exhibited mild spongiosis. Slight basal vacuolization was present in about 50% of the lesions. Epidermal atrophy and follicular plugging were absent. No epidermal basement membrane zone thickening was observed with a PAS stain.

A mononuclear cell infiltrate was densely aggregated around venules of the upper reticular dermis and more loosely arranged around venules of the papillary dermis. Perivenular aggregates of mononuclear cells were also regularly disposed about adnexa. In each case, intervascular and perivenular oedema along with oedema and vacuolization of venular basement membrane zones and endothelial cell swelling, were present. In the upper portion of the dermis nuclear debris was observed in seven cases.

Adjacent exposed skin. In adjacent exposed skin, only a few perivenular mononuclear cells within the upper dermis were present.

Weal (subject 3). In association with a slight perivenular mononuclear cell infiltrate in the upper dermis, perivascular, intervascular and venular basement membrane zone oedema was observed as well as endothelial cell swelling.

Immunoperoxidase studies

Anti-T cell antibodies stained most of the infiltrating mononuclear cells in papules of PMLE (Table 1). Staining was confined to cell borders of round cells that were present chiefly as dense nodular perivenular aggregates in the papillary and upper reticular dermis. Anti-T3 antibody stained 65–85% of infiltrating cells (Fig. 2). Furthermore, a combination of anti-T4B and anti-T8 antibodies stained the majority of mononuclear cells in all cases (Fig. 3) and were roughly equal to the total number of T4-positive and T8-positive cells counted on

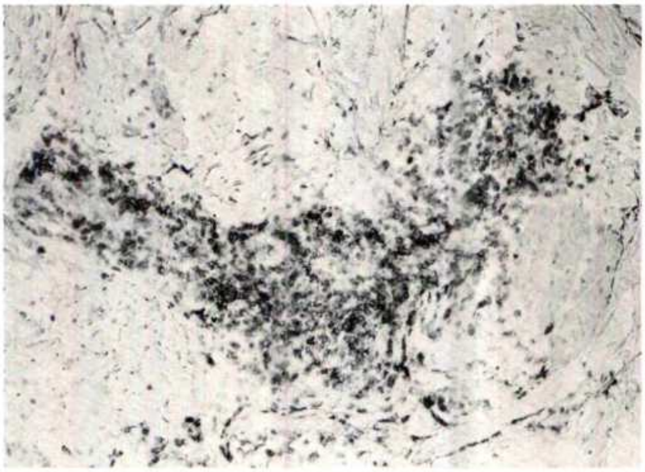

FIGURE 2. Frozen tissue section of a lesion of papular PMLE stained with anti-T3 antibody. A large number of T3+ cells are observed in a dense perivascular aggregate (× 128).

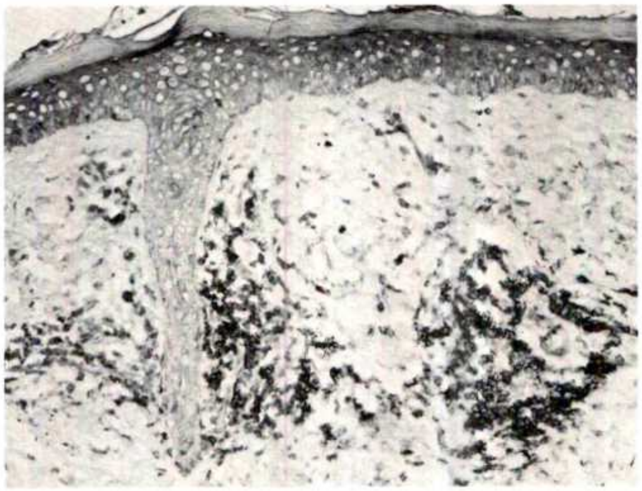

FIGURE 3. Frozen tissue section of a lesion of papular PMLE stained with a mixture of anti-T4B and anti-T8 antibodies. Most of the infiltrating perivascular cells exhibit cell surface staining (× 160).

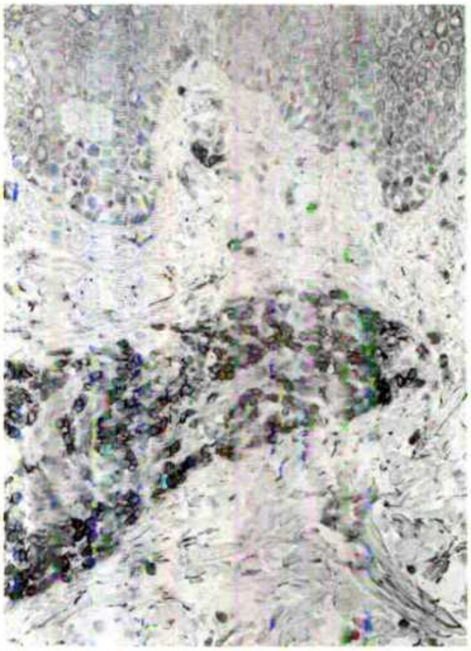

FIGURE 4. Frozen tissue section of a lesion of papular PMLE (subject 2) stained with anti-T4B antibody. A large number of T4+ cells infiltrate about vessels (× 205).

corresponding sections stained separately with anti-T4B and anti-T8 antibodies. Although T cells with cytotoxic/suppressor cell phenotype (T8-positive) predominated in four cases, two cases had a predominance of cells with helper/inducer phenotype (T4-positive), and two cases had roughly equal populations of both phenotypes (Figs 4 and 5). The relative proportions of the two T-cell phenotypes could not be correlated with the age of the individual lesion; however, in all cases in which the duration of photosensitivity exceeded 10 years a predominance of T8-positive cells was noted. A few cells within the epidermis stained with anti-T3, anti-T4, or anti-T8 antibodies in biopsy specimens of papules.

Epidermal dendritic and perivenular mononuclear cells stained with anti-T6 antibody in all lesions; apparent cytoplasmic and dendritic staining was observed. The number of T6-positive cells in the dermis was increased in five subjects as compared with control subjects; two of three subjects had increased numbers of dermal T6-positive cells in a papule as compared with adjacent exposed skin. In subject 3, a large number (about 30%) of the infiltrating mononuclear cells in both the papule (PMLE) and the weal (solar urticaria) were T6-positive (Table 1; Fig. 6). In contrast, the mean number of epidermal T6-positive cells in lesions (8·6 ± 5·0 cells/mm) did not differ significantly from that observed in adjacent exposed skin (6·8 ± 2·9 cells/mm).

Ia-positive (I2-reactive) cells, which exhibited cytoplasmic, dendritic, or peripheral (cell border) staining, represented 40–70% of the dermal infiltrate in papules, exceeding the number of T6-positive cells in all cases (Table 1, Fig. 7). However, anti-I2 antibody stained less than 30% (mean) as many epidermal dendritic cells as reacted with anti-T6 antibody; this finding was observed consistently in lesional, adjacent exposed and control skin. The mean number of epidermal Ia-positive cells was increased in papules (3·8 ± 1·0 cells/mm) as compared with adjacent exposed skin (0·8 ± 0·5 cells/mm). Endothelial cells and epithelium of sweat ducts (dermal and epidermal) were Ia-positive.

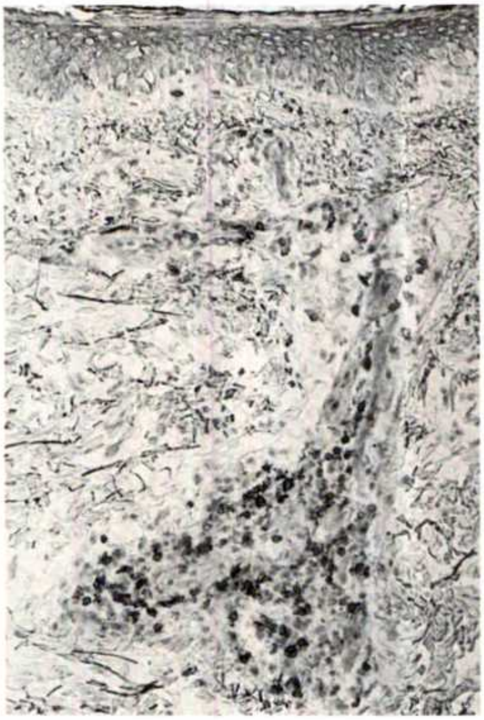

FIGURE 5. Frozen tissue section of a lesion of papular PMLE (subject 1) stained with anti-T8 antibody. About 50% of the infiltrating cells are T8+ (× 160).

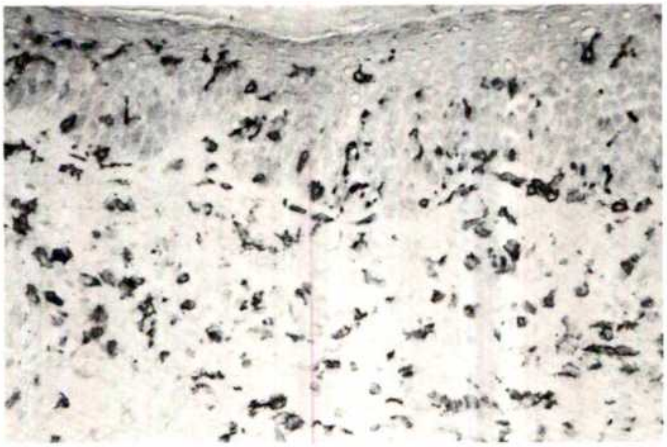

FIGURE 6. Frozen tissue section of a lesion of papular PMLE (subject 3) stained with anti-T6 antibody. Increased numbers of T6+ cells are present within the epidermis and dermis (× 205).

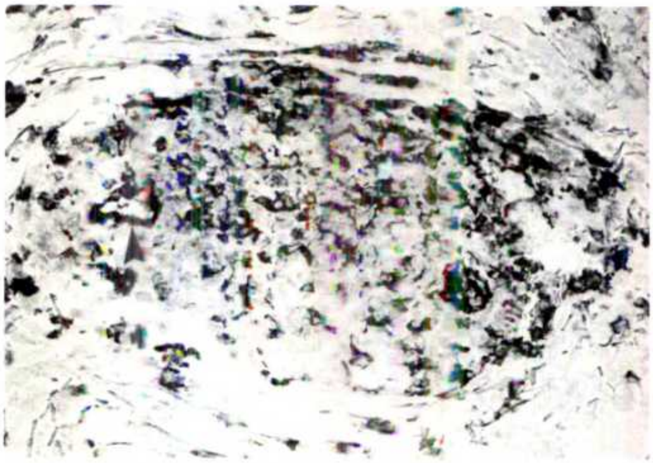

FIGURE 7. A papule of PMLE with Ia-reactive (Ia-positive) cells in a perivascular distribution. Endothelial cells are also Ia-positive (arrow) (× 205).

IgM was detected on rare dermal mononuclear cells in three skin lesions. Anti-M1 antibody stained cell borders of a small minority of infiltrating mononuclear cells. Adjacent exposed skin of subjects 2 and 3 contained a few dermal T6, Ia and T8-positive cells, but the numbers and cell phenotypes did not differ from those observed in normal skin of control subjects. Adjacent exposed skin from subject 1 had more T4-positive cells and T8-positive cells (T4/T8 ratio 5:3) than were observed in skin of control subjects.

DISCUSSION

A dense perivascular mononuclear cell infiltrate is a constant feature of PMLE (Wright & Winer, 1960; Epstein, 1980). The present study indicates that most of these infiltrating cells are T cells, which form dense perivenular aggregates without significant epidermotropism. Both major T-cell subsets, helper/inducer (T4-positive) and cytotoxic/suppressor (T8-positive) cells, are present in varying proportions in the infiltrate. The T8-positive subset appeared in greater numbers, albeit not strikingly, in four patients. The T4-positive subset formed the greater proportion of the infiltrate in two cases, one of which had a striking 7:1 ratio of T4-positive to T8-positive cells. In two cases, the T4:T8 ratio was approximately 1:1. The relative proportions of T4-positive and T8-positive cells could not be correlated with the clinical duration of the lesion. Study of sequential lesions in a subject with PMLE might show whether an orderly sequence of infiltration of the two major T cell subsets occurs.

In the majority of biopsy specimens of papules, increased numbers of dermal T6-positive (Langerhans/indeterminate) cells were present primarily in perivascular array. Subject 3, who had both papular PMLE and solar urticaria, was of particular interest since both the papule and weal exhibited the highest proportion of T6-positive dermal cells (30%). The relative predominance of T6-positive and T8-positive cells in both of his lesions does not, however, necessarily imply identical immune mechanisms in solar urticaria and PMLE.

As most of the inflammatory cells were T cells, and a large number of dermal Ia-reactive cells were identified, it is reasonable to assume that at least some of the T cells expressed Ia-antigen and therefore were in an activated state (Reinherz *et al.*, 1979).

As described previously (Muhlbauer *et al.*, 1982b), monoclonal anti-I2 antibody stained fewer epidermal Langerhans cells than did anti-T6 antibody, supporting the notion that an Ia-negative, T6-positive subset of Langerhans cells may exist. The finding of many more epidermal Ia-positive cells in lesions as compared with adjacent exposed skin suggests that more epidermal Langerhans cells express detectable HLA-DR antigen in papules of PMLE.

Although clinical observations suggest that PMLE is T cell-mediated, preliminary studies of lymphocytes *in vitro* have been conflicting (Horkay & Mészáros, 1971; Raffle, Macleod & Hutchinson, 1973). In one study (Jansen & Helander, 1976), homogenates of UV-irradiated and non-irradiated skin from patients with PMLE were incubated with autologous lymphocytes to test the hypothesis that PMLE involves a delayed reaction to antigen formed in the skin following UV radiation. No leukocyte migration inhibition was observed with either of the skin homogenates. Although their experiment did not support the above hypothesis, their samples contained mainly epidermis and antigenic material within the dermis may have escaped detection.

The present study provides evidence for a large number of perivascular T lymphocytes in PMLE. Possibly T cells are accumulating in response to antigen formed in a vascular or perivascular location following UV radiation. Langerhans cells present in the infiltrate may play a role in presentation of antigen to T lymphocytes (Silberberg-Sinakin & Thorbecke, 1980), which in turn proliferate and release lymphokines that attract additional lymphocytes to the tissue (Cohen *et al.*, 1973), damage blood vessels, and locally activate the clotting system (Rickles *et al.*, 1973; Rocklin, Bendtzen & Greineder, 1980; Muhlbauer *et al.*, 1982a). Accumulation of intervascular fibrin (Muhlbauer *et al.*, 1982a) and water may cause induration and form the clinically observed papule (Dvorak *et al.*, 1974). As both T-cell subsets were present in the infiltrate, it is possible that, in addition to a delayed type hypersensitivity response, cytotoxic lymphocytes (Cerottini & Brunner, 1974) could directly damage endothelial cells, producing the observed vascular injury.

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Immunohistochemical localization of laminin, type IV and type V collagen in basal cell carcinoma

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SUMMARY

Laminin, type IV and type V collagen were localized in twenty-five basal cell carcinomas (BCCs) by immunofluorescence. All basal cell carcinomas were separated from the connective tissue by a continuous basement membrane (BM) which always contained the three antigenic determinants. Nodular and cystic BCCs, but not pagetoid BCCs, contain linear and globular deposits of these BM components. The linear deposits probably represent BM debris that remains undigested and the globular deposits correspond to residual cytoplasmic fragments occurring during apoptosis. These results suggest that BM components are synthesized by epithelial cells and are secreted to polymerize at the dermo-epidermal junction.

The interface between epithelia and connective tissues is lined by a basement membrane that is a complex structure which includes the plasma membrane of basal cells, the lamina lucida, the basal lamina and a subbasal layer containing anchoring fibres, microfibril bundles and collagen fibres (Yaoita, Foidart & Katz, 1978). Several defined molecular components are known to participate in the composition of basement membranes, including the bullous pemphigoid antigen, laminin, type IV collagen and type V collagen (Briggaman, 1982).

There is little information concerning the nature and the fate of the basement membrane of epithelial neoplasms of the skin. We have reported that PAS-positive structures line basal cell carcinomas and that a similar material is present in globules inside regressing foci of the neoplasm (Franchimont *et al.*, 1982). Using immunofluorescence techniques, our preliminary results (Van Cauwenberge, Foidart & Lapière 1980), recently confirmed in a few patients (Weber *et al.*, 1982), suggested that macromolecules normally found in basement membranes were present in a continuous sheet around basal cell carcinomas and in globules within the

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neoplasm. The aim of this work was to investigate the biological significance of these findings in a larger group of patients and in different types of basal cell carcinomas.

METHODS

Clinical material

Twenty-five histologically confirmed basal cell carcinomas were studied in patients aged 65 ± 12 s.d. years. Their clinical and histological aspects corresponded to ten pagetoid, one reticular, ten nodular and four cystic basal cell carcinomas. After surgical excision, fragments collected at the periphery of the lesions were embedded in OCT compound (Lab Tek products division, Miles Laboratories, Inc. Naperville) and frozen in liquid nitrogen. Six micrometres thick sections were cut with an International Cryostat. Other samples were immersed in 70% ethanol, embedded in paraffin and 5 μm sections were reacted with antibodies to basement membrane antigens according to the technique described by Sainte Marie (1962).

Preparation of antibodies

Type IV collagen and laminin were purified from a murine tumor producing a basement membrane matrix (Orkin *et al.*, 1977; Timpl *et al.*, 1978; Timpl *et al.*, 1979). Type V collagen was purified from human placenta (Burgeson *et al.*, 1976). Antibodies were raised in rabbits as described by Foidart & Reddi (1980). Affinity purified antibodies to type IV collagen, laminin and type V collagen were prepared from their respective antisera by cross immunoadsorption (Nowack *et al.* 1976). Specificity of the antibodies was assessed by radioimmunoassay (Scheinman, Foidart & Michael, 1980), enzyme-linked immunoassay (Rennard *et al.*, 1980) and blockage of immunofluorescence or competition studies as previously reported (Yaoita *et al.*, 1978).

Indirect immunofluorescence studies

Sections were rinsed for 5 min in phosphate-buffered saline (PBS). Affinity purified antibodies (20 μg/ml PBS) were then applied to tissue sections and incubated at room temperature in a moist chamber for 30 min. Sections were washed in five changes of PBS for 5 min each and dried.

Sections were then incubated with fluorescein isothiocyanate conjugated (FITC) goat antibodies to rabbit IgG for 30 min in a moist chamber at an antibody concentration varying between 50 and 150 μg/ml. The sections were finally washed three times with PBS, covered with a solution of 90% gylcerin and 10% PBS, mounted and viewed under a Zeiss epi-illuminated fluorescence microscope. Purified rabbit antibodies to ovalbumin (50 μg/ml PBS) and normal rabbit IgG 20–50 μg/ml PBS were used as controls.

After IFF studies, sections prepared according to Sainte Marie (1962) were counterstained with haematoxylin eosin or periodic acid Schiff (PAS) in order to define more precisely the histological structures responsible for binding of the antibodies.

RESULTS

Type IV, type V collagen and laminin were present in all normal basement membranes at the dermal epidermal junction, and around blood vessels, sweat glands, hair follicles and smooth muscle cells.

At the site of basal cell carcinomas, three patterns of distribution of the immunoreactants were

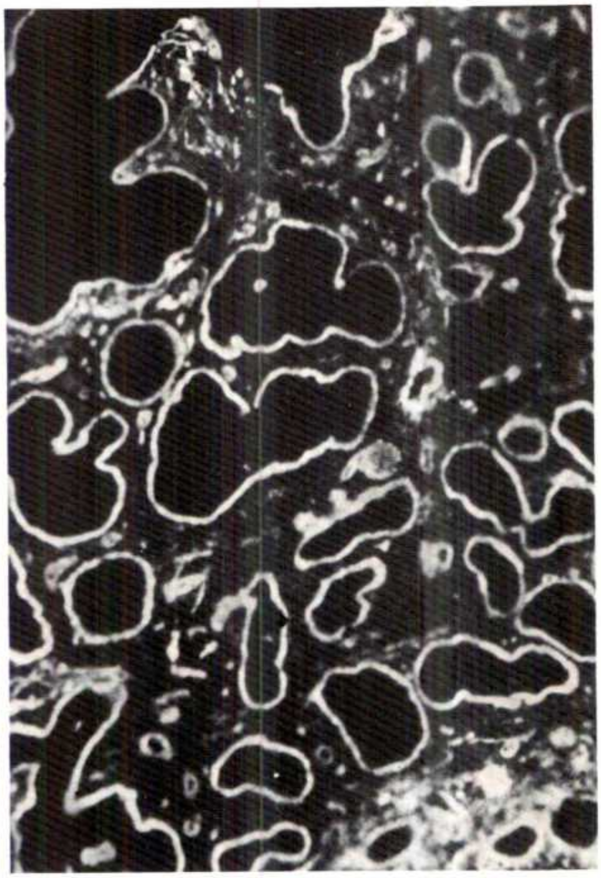

FIGURE 1. Immunolabelling of a basal cell carcinoma produced by reacting antibodies raised in the rabbit against laminin and revealed by a fluorescein labelled goat anti-rabbit IgG. Basement membrane around epithelial nests and vessels is shown.

disclosed. The first pattern was seen in all basal cell carcinomas at the junction between the neoplasm and the dermis. Linear deposits for all the immunoreactants were observed displaying the same immunohistochemical characteristics as those of the normal basement membrane (Fig. 1). They were continuous and sometimes uneven in thickness. Linear fragments in continuity with the peritumoral basement membrane were sometimes present outside the neoplastic nodules in the remodelled surrounding connective tissue (Fig. 2). They were distinct from the basement membrane of the numerous vessels (Fig. 3). The second pattern consisted of linear, sometimes corrugated, deposits located inside the neoplasm and connected to the peritumoral basement membrane (Fig. 4). It corresponded to the irregular infolding of this membrane. The third pattern was present in several basal cell carcinomas where types IV and V collagen and laminin were localized inside the tumour nests in a granular and patchy distribution. Their numerical density and their apparent size increased towards the centre of the neoplastic nests (Figs 4 and 5). A diffuse labelling was visible in the most peripheral layers of neoplastic cells (Fig. 5). When the same sections were counterstained in standard microscopy, these

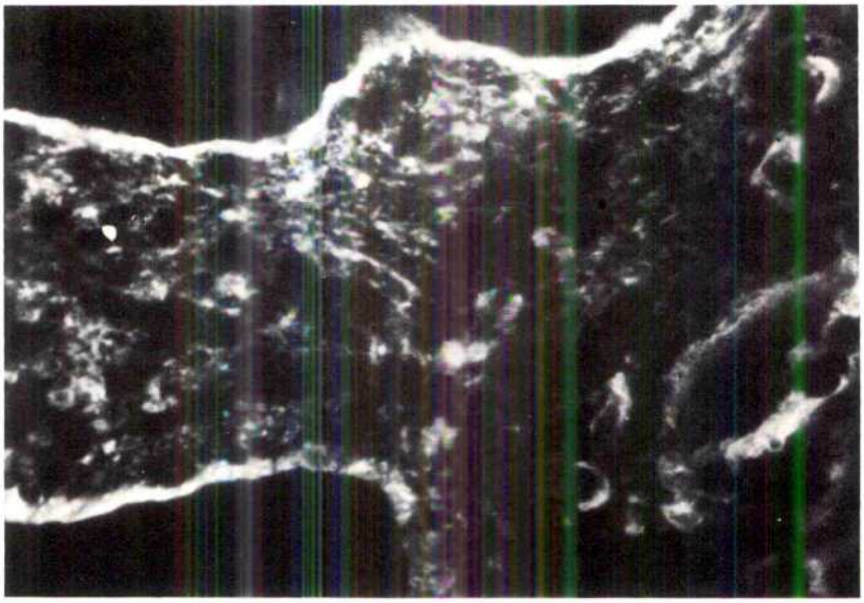

FIGURE 2. Immunolabelling of basal cell carcinoma with antibodies raised against collagen type IV. The diffuse labelling in the connective tissue between two adjacent nests of basal cell carcinoma is illustrated.

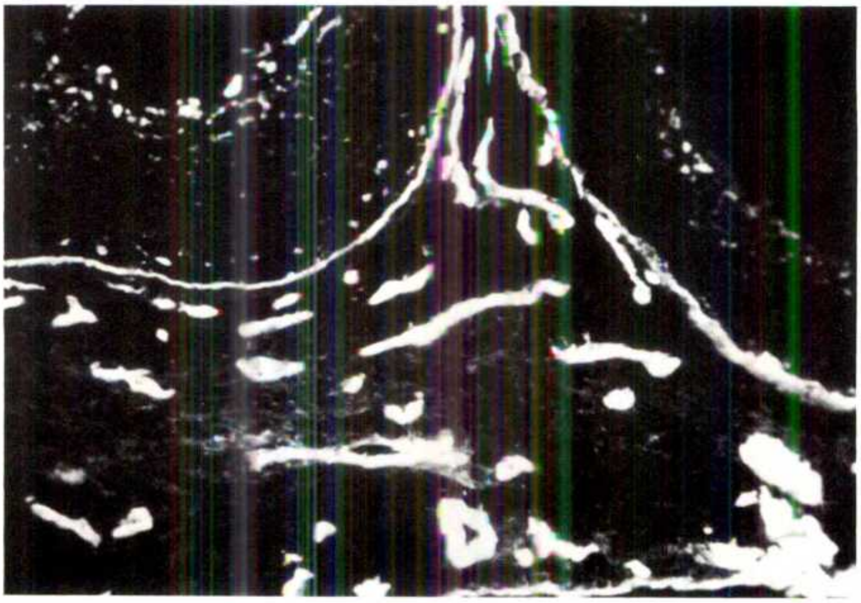

FIGURE 3. Immunolabelling of a cystic basal cell carcinoma produced by the anti-laminin antibody. It demonstrates the aspect of the lamina densa around the neoplastic cells and the blood vessels. Immunoreactive bodies are present inside the nest of basal cells.

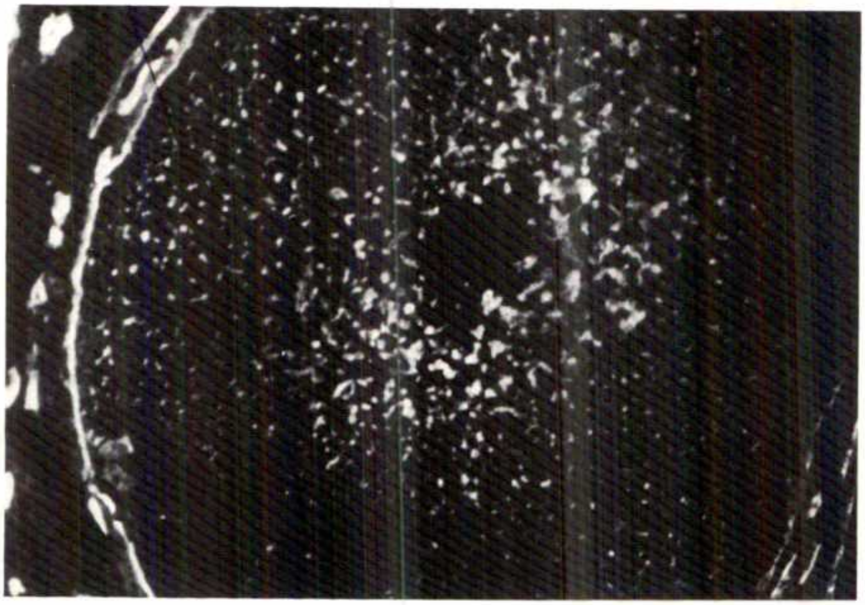

FIGURE 4. Immunolabelling of a cystic basal cell carcinoma produced by type IV collagen antibody. Immunoreactive bodies are present inside the neoplastic nests, some connected to the basement membrane in a gradient of increasing density towards the centre of the necrotic cavity.

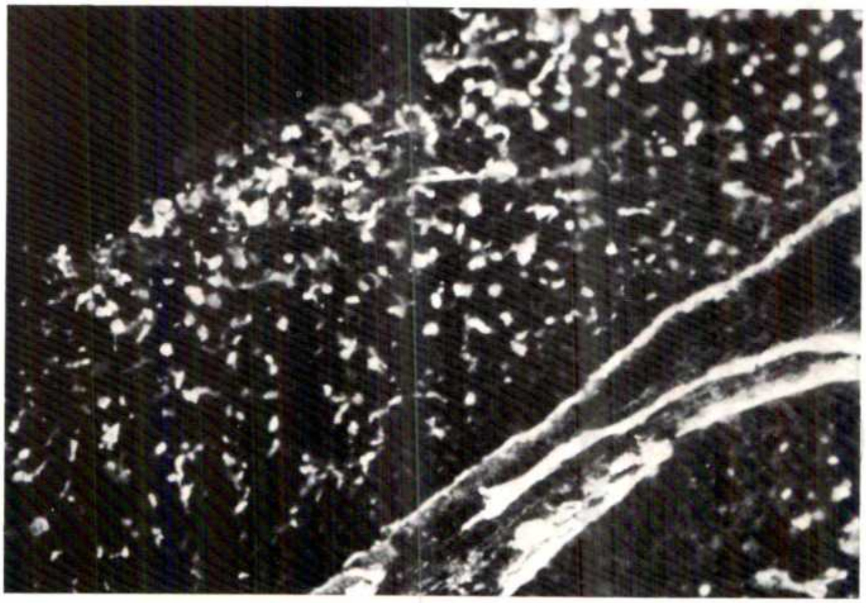

FIGURE 5. Immunolabelling of two adjacent nests of a cystic basal cell carcinoma (as in Fig. 3). It illustrates the increasing density of globules and immunoreactive material towards the centre of the tumour. It also demonstrates the diffuse labelling of the outer layers of tumour cells.

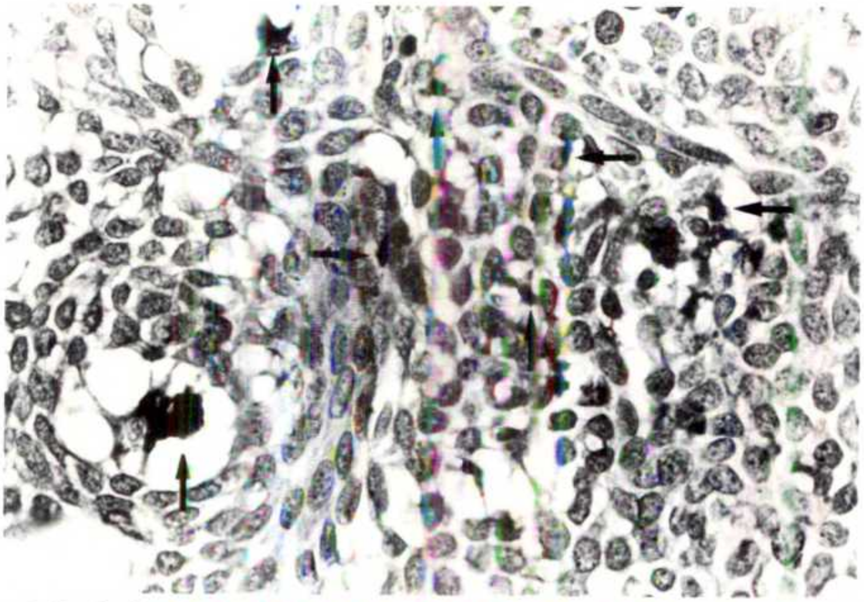

FIGURE 6. Basal cell carcinoma in which nucleated neoplastic cells and PAS positive apoptotic bodies (arrows) are recognized (PAS hemalun staining).

immunofluorescent bodies were found to be eosinophilic and PAS positive (Fig. 6). These bodies were most abundant in nodular cystic basal cell carcinomas (Figs 3, 4 and 5), less frequent in pagetoid basal cell carcinomas and almost absent in the reticular basal cell carcinoma (Figs 1 and 2).

DISCUSSION

Basement membrane is a morphological entity composed of the defined macromolecules type IV and V collagen and non-collagenous glycoproteins, including laminin and bullous pemphigoid antigen (Yaoita *et al.*, 1978; Briggaman, 1982). As observed by transmission electron microscopy, basement membrane which delineates basal cell carcinomas is irregular in thickness (Kobayasi, 1970; Hashimoto, Yamanashi & Dabbous, 1972; Pierard, Hermanns & Lapière 1974). Discontinuities detected by transmission and scanning electron microscopy are too small to be observed by immunofluorescence (Pierard *et al.*, 1974; Kint, 1976). Our study suggests that basement membrane around basal cell carcinomas contains all the antigens normally present at the normal dermo-epidermal junction. Kimura *et al.* (1980) have also shown the presence of bullous pemphigoid antigen. An asynchronous deposition of the constituent macromolecules has been reported in other models where basement membrane is being formed (Hintner *et al.*, 1980; Stanley *et al.*, 1981). This is not the case in basal cell carcinomas in which the concurrent presence of the three macromolecules in linear and continuous deposition suggests their simultaneous synthesis with no spatial or temporal dissociation. Since basal cell carcinomas consist of the juxtaposition of nests constantly in proliferation and regression (Franchimont *et al.*, 1982) this investigation corresponds in fact to a time-course study of the development of the neoplasm.

In some foci the basement membrane presented linear extensions outside and inside the neoplastic buds. Those outside the neoplasm could represent the so-called reduplication of basement membrane that accompanies epithelial regression, as seen in lichen planus and lupus erythematosus. We interpret their presence as remnants of basement membrane after partial regression of the neoplasm. Those inside and closely associated with the basement membrane are invaginations that occur during the growth of the neoplasm. The three basement membrane antigens were also disclosed in globular form inside most foci of nodular and cystic basal cell carcinomas and were more concentrated in the centre of neoplastic nodules near the necrotic cavity. These remnants probably persist in the absence of the enzyme capable of ensuring their proteolytic digestion. These latter deposits are almost absent in the reticulated and pagetoid types of basal cell carcinomas revealing distinct biological features for these different histological types of the neoplasm.

In addition to the undigested breakdown products of established basement membrane, some of the globular bodies may represent residual undegraded cytoplasmic fragments resulting from apoptosis and cellular death (Curson & Weedon 1979; Weedon, Searle & Kerr 1979). The identification of the three basement membrane proteins in these presumed apoptotic bodies indicates that the constituent structural proteins were synthesized but not secreted.

In recent years it has become apparent that many neoplasms may gain new biosynthetic capacities while they retain some properties of the cells from which they derive. The parallel expression in basal cell carcinoma cells of the genes coding for the three macromolecules specific for basement membrane, suggests that their synthesis is a physiological characteristic of basal keratocytes unrelated to cancer. It supports the hypothesis that epithelial cells are responsible for the synthesis of laminin, type IV and type V collagen. The fact that granules containing these three macromolecules are also found inside the neoplasm suggests that synthesis of basement membrane macromolecules has taken place in cells that have lost contact with the connective tissue. It would indicate that a close interaction between mesenchymal structures and epithelial cells is not required for synthesis but may be needed for secretion and organization. This seems to be specific for basement membrane components because interstitial collagens and procollagens were not found inside the nests of basal cell carcinomas. Almost identical results were obtained in cylindroma (personal unpublished data).

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A local potent glucocorticosteroid decreases the induction of galactosylhydroxylysyl glucosyltransferase in suction blisters but has no effect on basement membrane structures

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SUMMARY

The effects of local glucocorticosteroid treatment on collagen biosynthesis and basement membrane components were studied in suction blisters in human abdominal skin. Pretreatment with clobetasol-17-propionate, applied three times a day for 4 days, did not affect the activity of galactosylhydroxylysyl glucosyltransferase (GGT) in fresh blisters but post-blistering treatment for 3 days with the steroid markedly inhibited the increase of this enzyme activity during the initial phases of re-epithelialization. The GGT activity was over 50% lower in steroid-treated blisters compared with control values. Protein concentrations and blister fluid volumes were also significantly decreased in healing 3-day blisters after steroid treatment. These results suggest that local glucocorticosteroid decreases either the synthesis of GGT or its release from the tissue into the blister fluid.

The treatment did not affect the blister histology nor the early process of re-epithelialization. Immunohistochemically, type IV and V collagens and laminin of the basement membrane zone were similarly located in blisters of steroid-treated and placebo-treated skin, suggesting that local glucocorticosteroid does not affect the integrity of the basement membrane.

Suction blisters have been used to study the epidermal-dermal basement membrane and the epithelialization process in standard wounds on animal (Krawczyk, 1971; Pang, Daniels & Buck, 1978; Devitt *et al.*, 1978) and human (Ortonne *et al.*, 1981; Saksela *et al.*, 1981) skin. Recently we have shown that during re-epithelialization of the blister base the activity of GGT, an enzyme involved in collagen biosynthesis, increases markedly owing to its mainly local production (Oikarinen *et al.*, 1982). Because glucocorticoids tend to decrease both collagen

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synthesis (Rokowski, Sheehy & Cutroneo, 1981) and the activities of enzymes in collagen biosynthesis (Cutroneo & Stassen, 1975; Oikarinen, 1977; Risteli, 1977; Oikarinen & Hannuksela, 1980) we were interested in studying the effects of a highly potent topical glucocorticosteroid on the activity of GGT in the 'wound' fluid of human skin blisters. We also wished to see whether or not the basement membrane between the new epidermis and the dermis, as well as the pre-existing basement membrane remaining adhered to the blister bed, would be affected by steroid treatment. The components of basement membranes were studied using specific antibodies to type IV and V collagens and laminin.

METHODS

Subjects and treatment

Eight male volunteers (aged 19–22 years) received local 0·05% clobetasol-17-propionate (Dermovate® ointment, Glaxo) treatment without occlusion three times a day on one side of the abdominal skin in a square 9 × 9 cm, and the ointment base without corticosteroid in a similar square on the other side. Pretreatment was started 4 days before blister induction; the treatment was continued for 3 days after blistering.

Suction blisters, samples and assays

Suction blisters (5 mm in diameter) were induced both on the steroid-treated and on the placebo-treated areas of the abdominal skin as described elsewhere (Kiistala, 1968). All the samples were taken in accordance with the Declaration of Helsinki. For total-protein and GGT assays, blister fluid samples were withdrawn by a Mantoux syringe immediately after blister formation, and again 3 days later. For each sample ten to twenty intact blisters were emptied and pooled. The remaining intact blisters were shielded by perforated plastic caps until they were emptied at the second sampling. Pooled samples were frozen and kept at −20°C for a maximum of 3 months until assayed.

The protein concentration in the blister fluid was determined by the method of Lowry *et al.* (1951). GGT activity was assayed in pooled blister fluid by the method of Myllylä, Risteli & Kivirikko (1975) as modified by Anttinen (1977).

For immunohistochemical studies, four suction blisters together with the surrounding skin were excised (both from the steroid- and placebo-treated sites) under local anaesthesia. Such samples were taken immediately after blister formation and again 3 days later: they were frozen immediately and kept at −20°C for up to three months. The samples were cut into 7 μm cryostat sections (Cryo-Cut, American Optical Corporation) on albumin-coated slides. The sections were air-dried for 30 min, fixed for 5 min in ice-cold methanol, and washed three times for 5 min each time in phosphate-buffered saline (PBS), pH 7·4. The sections were then incubated for 10 min with normal goat serum, washed twice for 5 min in PBS and incubated for 30 min with purified, highly specific collagen antibodies (100 μg/ml PBS) or with control sera. They were then washed in PBS five times, 5 min each time. Fluorescein-conjugated goat IgG (1:40 dilution, Dako) to rabbit IgG was applied for 30 min; this procedure was followed by washing in three changes of PBS. The sections were then mounted in glycerol PBS (1:1 by volume) and examined by epifluorescence microscopy (Orthoplan, Leitz).

Type IV collagen and laminin were purified from murine EHS sarcoma tissue (Orkin *et al.*, 1977; Timpl *et al.*, 1979) and type V collagen from human placental membranes (Sage & Bornstein, 1979). Antibodies against the collagen types and laminin were prepared in rabbits, and were cross-absorbed and further purified by immunoaffinity chromatography (Timpl,

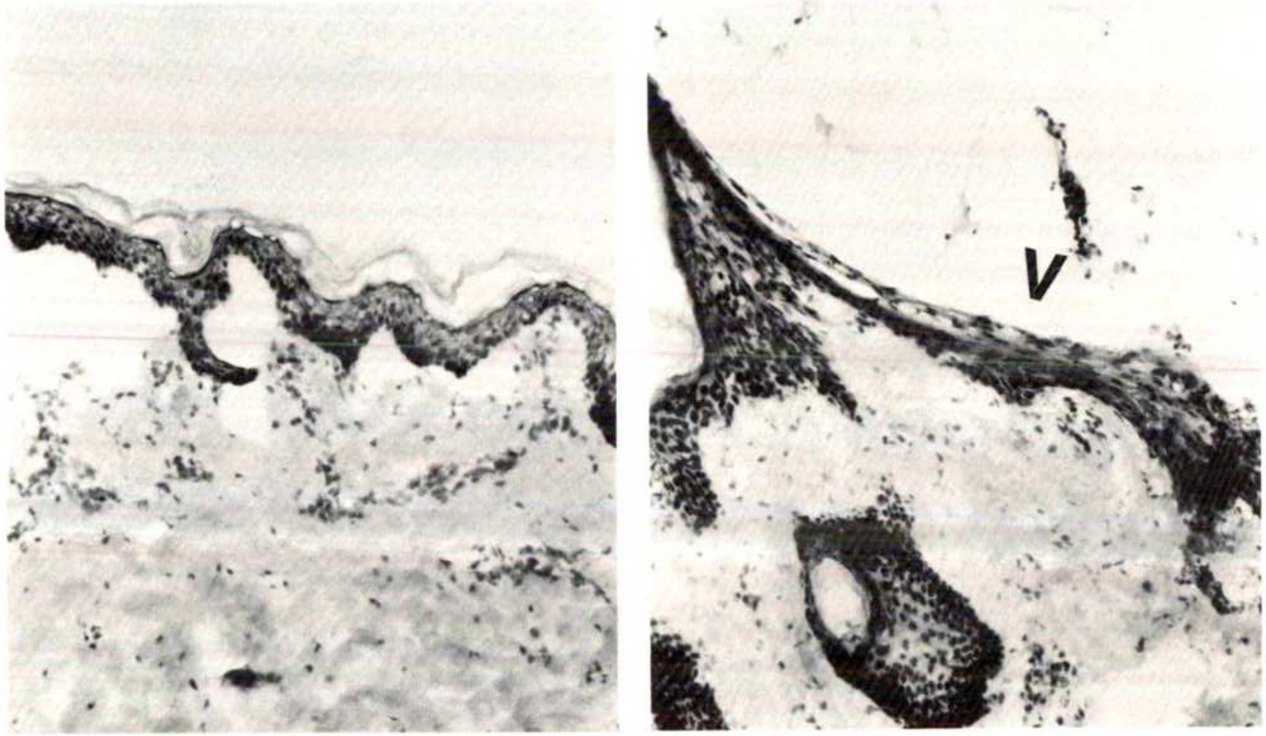

FIGURE 1. Histological picture of fresh blister (a) and day 3 blister after Dermovate® treatment (b). (a) The entire epidermis forms the roof of the blister. In control blisters the finding was similar. (b) The arrow indicates the regenerating new epidermis, and there are some cells in the blister cavity. Hematoxylin eosin × 90 in both (a) and (b).

Wick & Gay, 1977). The specificity of the purified antibodies was established by immunofluorescence blocking and by passive haemagglutination. For the histological analysis samples were stained with haematoxylin and eosin.

Statistical analysis

Student's *t* test was used to calculate the statistical significance of the differences between the means.

RESULTS

Histological and immunofluorescence findings

The microscopic separation of the epidermis appeared similar in Dermovate®-pretreated and placebo (ointment base) pretreated skin (Fig. 1a). The whole epidermis formed the blister roof. Re-epithelialization started from the blister margins (Fig. 1b) in both steroid-treated and placebo-treated skin. At day 3 roughly a quarter of the blister bed was covered by newly-formed epithelial tongues in both steroid-treated and placebo-treated skin (not shown), and no clear difference could be found in the speed of epidermal repair either by clinical or by morphological observation. However, the gross appearance of the steroid-treated blisters was more flaccid.

Laminin and type IV and V collagens were found in non-blistered areas of the epidermal-dermal junction and in the walls of dermal blood vessels in both steroid-treated and placebo-treated skin (data shown for type V collagen, Fig. 2). In newly-induced blisters, type IV and V collagens and laminin were found in the denuded junction zone over the blister base and along the

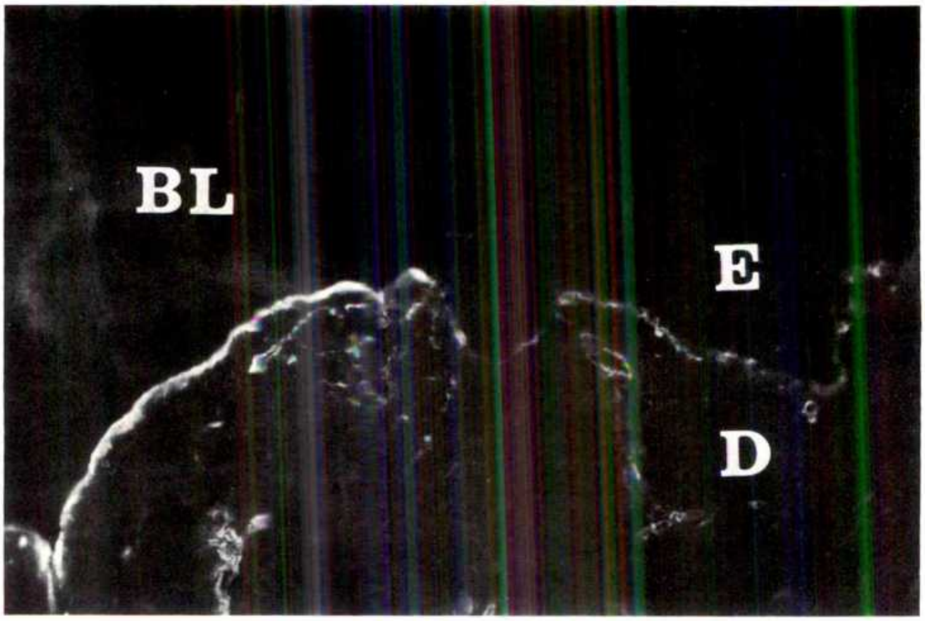

FIGURE 2. Indirect immunofluorescence staining of human skin blister using affinity-purified type V collagen antibodies. Skin was pretreated with the ointment base and the sample was taken immediately after blister induction. Notice the linear staining in the base of the blister and at the epidermal-dermal junctions. Some staining is also found in the blood vessels. (Magnification × 210) Epidermis (E); dermis (D); blister (BL).

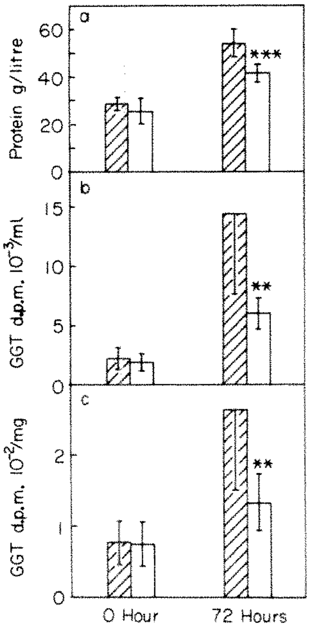

FIGURE 3. Protein content and GGT activity in blister fluid in fresh (0–h) and day 3 (72-h) blisters in Dermovate®-treated (□) and placebo-treated (■) skin. (a) Protein content in blister fluid (g/100 ml). (b) GGT activity in blister fluid expressed as d.p.m. per ml of blister fluid. (c) GGT activity in blister fluid expressed as d.p.m. per mg of blister protein. The values are the mean ± s.d. $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$.

underlying vessels. Type IV and V collagens were not found in the separated epidermis, but in some places laminin was observed along the lower margin of the separated roof. These findings were identical in blisters of both steroid-pretreated and placebo-pretreated skin. The changes were also identical during the healing process.

Blister fluid volume, protein content and GGT activity

The mean volume (5 mm blisters) in fresh control blisters was 43 ± 8 µl (mean ± s.d.) and in steroid-pretreated blisters 41 ± 10µl. At day 3, post-blistering, the mean volume of control blisters was 29 ± 10 µl and that of steroid-treated blisters 13 ± 4 µl; the difference was statistically significant ($P<0{\cdot}001$).

Total protein concentration in fresh steroid-pretreated blisters was slightly lower than in control blisters. The difference was not statistically significant. At day 3, the protein concentration was 22% lower in steroid-treated than in control blisters ($P<0{\cdot}001$; Fig. 3a). GGT activity in newly-formed steroid-pretreated blisters was slightly lower than in control blisters (statistically not significant), but at day 3 with continuous treatment, GGT activity was significantly lower ($P<0{\cdot}01$) in steroid-treated than in control blisters. This difference was noted whether calculated per fluid volume in microlitres or per protein concentration of the blister fluid (Fig. 3b and 3c).

DISCUSSION

In the first part of this study clobetasol-propionate ointment was applied for 4 days on intact skin, preceding blister production. This pretreatment affected neither the anatomical plane of epidermal separation nor the distribution of the examined basement-membrane-zone components of the underlying blister bed. This finding is not unexpected, since after relatively short-term administration corticosteroids have only a minor influence on mature tissues with relatively low synthetic activity. The slight trends toward lower protein and GGT concentrations in fresh blisters on the steroid-treated skin might parallel the phenomenon of corticosteroid-induced skin thinning. Tan, Marks & Payne (1981) have recently observed an early onset of dermal thinning following 2 days treatment with creams containing 0·05% clobetasol propionate. This reversible thinning was of the order of 5%, and was more marked after longer periods of treatment.

In the second part of this study applications of the corticosteroid were continued on blistered skin for a period of 3 days. The treatment induced a definite involution of blisters and a relative decrease in protein concentration. Indeed, in steroid-treated skin the blister volume was less than one-half of that in control sites, and correspondingly the protein concentration was almost a quarter lower in steroid-treated blisters. Newly-induced suction blisters are known to have a relatively low protein concentration, in the order of 40% of the serum level, but there is a progressive increase in older blisters. Values as high as 80% of the serum level can be attained in old blisters (Kiistala, 1976; Oikarinen *et al.*, 1982). The exchange of proteins from the circulation into this extravascular compartment is relatively rapid (Worm, 1981), and the inflow and elimination rates of protein-bound drugs can be varied by grading the inflammatory stimulus in suction blisters (Schreiner *et al.*, 1978).

Many actions have been attributed to corticosteroids which may contribute to their anti-inflammatory properties, e.g. the reduction of vascular permeability leading to diminished plasma leakage into the extravascular tissues. This study shows that the anti-oedematous and anti-exudative properties of local corticosteroids can be quantified by the use of suction blistering, which is one of the few experimental models applicable to man for the study of inflammatory and therapeutic effects.

The role of GGT during regeneration is not known. It is important for the proper glycosylation in post-translational modification of collagen biosynthesis and reflects the activity of collagen biosynthesis (Kivirikko & Myllylä, 1979). The situation is, however, more complex because the enzyme is also found in the cells which are not actively synthesizing collagen, e.g. inflammatory (Myllylä & Seppä, 1979) and epidermal cells (Oikarinen *et al.*, 1982). So it is possible that the lowered increase in GGT activity in old blisters reflects a decreased response of inflammatory and epidermal cells to regeneration stimulus. Another reason may be that glucocorticoids inhibit or decrease the release of GGT from surrounding tissues to the blister cavity. Glucocorticoids decrease the synthesis of prolylhydroxylase (Cutroneo & Stassen, 1975; Oikarinen, 1977), an enzyme which is involved in collagen synthesis. It is not known if glucocorticoids also decrease the synthesis of GGT but this is probable because in animal tissues (Risteli, 1977) and cells (Oikarinen, 1977) glucocorticoids decrease the activity of GGT without inhibiting the enzyme reaction itself (Oikarinen, 1977). Clobetasol propionate is clinically the most potent corticosteroid available, and has been shown to possess *in vitro* a high binding affinity to cytosol of both human keratinocytes and fibroblasts (Ponec, Kempenaar & Dekloet, 1981). Although in our study it was observed to have a marked inhibitory effect on GGT activity, it did not have any noticeable inhibitory effect on the re-epithelialization process. During longer follow-up periods subtle differences might be observed, but on clinical grounds both steroid-treated and control blisters have apparently identical new epithelium over their floors by day 7. Our results are thus in agreement with observations made on epidermal regeneration of suction-blistered animal skin treated with i.c. dexamethasone; this treatment did not retard epidermal migration, although the DNA synthesis and post-blistering acanthosis of the blister margin were diminished (Devitt *et al.*, 1978).

The present study shows that corticosteroids do not have any clear influence on pre-existing basement membrane components in the blister area, such as laminin and type IV and type V collagens. These components were not increased under the new epithelium either. This finding is explained by the fact that laminin and type IV collagen appear relatively late under premature migrating epidermis (Stanley, Alvarez, Bere, Eaglstein & Katz, 1981). Suction blistering seems not to be a good model for the study of their early appearance, since in this process the basement membrane zone is not lost.

In this study we observed laminin foci also separated the epidermal rim, a finding in agreement with other authors (Saksela *et al.* 1981) but in disagreement with our own earlier report (Oikarinen *et al.*, 1982). One explanation for this apparent ambiguity might be that there are minor variations in separation owing to the complexity of the junction zone around the epidermal appendages, resulting in some variability in epidermal stripping. Thus, minor defects in the ultrastructural basal lamina are not unusual after suction separation (Ortonne *et al.*, 1981), and in animal skin even deeper breaks may be found (Levine *et al.*, 1982).

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Mast cell numbers in incisional wounds in rat skin as a function of distance, time and treatment

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SUMMARY

The dynamic changes in skin mast cell (MC) numbers around incised wounds were studied, using experimental designs amenable to multiple analyses of variance. Sixty-four Wistar albino rats were shaved in the interscapular region, wounded or not wounded, and then killed 2 or 10 days later. During this period, the rats were exposed continually to a cold (2°C) or control (20°C) climate and treated daily over the shaved region with either tap water or a weak sulphuric acid (pH 3·5) solution. The MCs within five adjacent fields of the wound or the control reference and within the superficial and deep halves of the skin were counted (at × 400). The greatest decrease in MC numbers occurred within about 700 μm of the wound. Whereas the paucity of MCs within the wound region was evident at 2 days, near-normal levels were achieved by day 10. Cold exposure produced little effect, but MCs responded differently to the water and acid treatments as a function of distance and skin depth.

Several authors (Holczinger & Devenyi, 1954; Wichmann, 1955; Boyd & Smith, 1959; Reynolds *et al.*, 1963; Matsuda & Kitamura, 1981) have implicated the skin mast cell (MC) in wound healing. The disappearance and reappearance of these multipotent cells around incisions have been reported by Wichmann (1955) and by Boyd & Smith (1959).

Experiments in which MCs have been counted as a function of the distance from the wound (e.g. Holczinger & Devenyi, 1954) have contained sampling problems or have not been amenable to clear statistical analysis. No study has employed experimental designs that would allow the detection of possible interactions between post-wound time, various treatments and distance from the wound. The use of statistical procedures that simultaneously employ temporal variables and distances and depths within the skin could allow the detection of dynamic changes in MC numbers.

The present study was designed to determine the effects of incised wounds upon MC

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numbers within the two major levels of the skin as a function of distance and time. To further test the practical possibility that MC numbers in wounded skin might respond differently from MCs in non-wounded skin to environmental influences, two other treatments were selected: the pH of fluid applied to the wound and the ambient post-wounding temperature.

Both ambient temperature and the pH of adjacent tissue have been reported to influence wound healing. Humid conditions at the wound promote epithelialization (Osment, 1975; Sciubba *et al.*, 1978) while cold environments (2°–3°C) decrease the rate of healing (Filston & Vennes, 1968) and warm temperatures (30°C) facilitate healing (Cuthbertson & Tilstone, 1967). The pH of wounded tissue is known to shift from acidic to basic levels during the repair process (Reynolds *et al.*, 1963). Granulocytes and fibroblasts are very sensitive to lower ambient pH (Peacock & Van Winckle, 1970; Silver, 1980). In fact, pH gradients have been suggested as a major stimulus for wound repair (Silver, 1980).

METHODS

Sixty-four male albino Wistar rats obtained from Woodlyn Laboratories (Guelph, Ontario) were used as subjects. In experiment I (thirty-two animals), the subjects were 60 days of age (mean wt 268 ± s.d. 11·7 g) while in experiment II (thirty-two animals), the subjects were 90 days of age (mean wt 350 ± s.d. 20 g) at the beginning of the treatments.

From 1 week before the experiment until its end, all rats were housed singly in metal wire cages. Purina rat chow and water were available *ad libitum*. The following colony conditions existed: temperature 23° ± 1°C and LD cycle 12 : 12.

On the day of the experiments, the animals were randomly assigned according to a 2 × 2 × 2 factorial design (three-way analyses of variance). For both experiments I and II, the animals were allocated to one of two wound treatments (wound-control versus wound) and to one of two kill times (2 days versus 10 days). The third treatment was different for the two experiments. In experiment I, the animals were allocated to either the tap water (control) or acidic pH condition while in experiment II the rats were assigned to either the control or cold environment.

On the day of the wound or wound-control treatments, each animal was injected i.p. with 40 mg/kg of sodium pentobarbital (Somnitol) and 0·4 cc of atropine sulphate. Rectangular-shaped areas of about 3·5 cm by 2·5 cm were produced in the interscapular regions of all animals by an electric razor. Animals in the control-wound group were stroked with a blunt instrument over the shaved skin. For the wound groups, 1·5 cm length incisions were made (through the skin to the underlying fascia) at an angle perpendicular to the long axis of the rat by a small pair of dissection scissors (experiment I) or a razor blade (experiment II). The wound was held closed manually or with tape for about 15 min before the animals were placed in plastic recuperation cages within the surgery room.

In experiment I, the animals were returned to the home cages in the colony. Half the numbers of rats from each of the treatment conditions were treated daily (starting 15 min after shaving) with either tap water (pH = 6·4) or weakly acidic dilute solutions of sulphuric acid (pH = 3·5); these solutions were applied at room temperature and made fresh every third day. The fluids were administered with saturated (dripping) cotton puffs applied over the shaved areas for 15 s so that the areas were well soaked. Fresh puffs were used for each animal and the order in which the solutions were applied was altered daily.

In experiment II, 6 h after the injection of the anaesthetic, animals were taken to a room containing a walk-in environmental chamber. Half the animals were maintained in the chamber (2° ± 1°C) while the other animals (temperature controls) were placed outside (20° ± 1°C).

Animals were maintained in similar single wire cages and given food and water *ad libitum*. Both groups were given a LD 12:12 regime similar to the initial colony conditions.

Either 2 or 10 days after the wound (or no-wound) treatments, each rat was weighed and killed by chloroform. Because of the hair growth in the day 10 groups, the area around the wound was shaved carefully. Each shaved area was then carefully removed and placed immediately between two glass slides in a tray of E:F:A (90 parts 80% ethanol:5 parts 30% formaldhyde:5 parts glacial acetic acid) in order to reduce curling. After a period of 10 min, the tissues were then placed into a separate jar of E:F:A for 24 h. Following dehydration, clearing and infiltration (Persinger, 1980; 1981) tissues were embedded in paraffin blocks. The blocks were sectioned at 6 μm in a plane perpendicular to the surface of the skin (and to the long axis of the incision). Five serial sections for every 100 sections were stained with toluidine blue for mast cells. In experiment I, other sections were stained with fuchsin degenerative and Verhoeff elastin stains (Humason, 1972).

Mast cells, as defined by both metachromasia and morphology, were counted at × 400 within the five adjacent fields from the wound and within the two adjacent fields of the upper and lower halves of the skin (5 × 2 = 10 fields). Optimal sections (minimal histological artifacts) at the level that most demonstrated the wound were selected. As an extra precaution (in expermiment I) since there was some overlap between the upper and lower fields of some sections, the average dermal depth of each tissue (section) was determined by a Trisimplex projector for later analyses of covariance.

In experiment I, the eight fields on one side and the eight fields on the other side of a wound (which was aligned in the middle of the two central fields) were counted and averaged for MCs. In experiment II, the ten fields were counted for only one side of the wound, but two separate sections (separated by 100 μm) were counted and averaged. For the control (no-wound) tissues, comparable regions in the middle (experiment I) or the edge (experiment II) of the tissue were selected as the central two fields. All tissues ($n = 32$) were sectioned, stained and counted for MCs. One tissue was lost in experiment II ($n = 31$).

Five-way analyses of variance with two factors repeated (two wound conditions, two kill days, two specific treatments, five fields from the wound and two fields of skin thickness) were completed by BMD statistical software using a DEC2020 System computer. Non-repeated measure analyses and checks for numerical properties of the data were verified using SPSS software.

Since some of the MC populations per field exceeded skewness values of 1·0, square roots of the MC numbers were also computed for both experiments. These data transformations reduced all skewness values to less than ± 0·9. Five way analyses of variance plus covariance for either body weight, change in body weight, surgical sequence, tier in the home cages, tier in the experimental cages, or thickness of tissue (experiment I only) were completed for the absolute and square root data.

RESULTS

The means and standard deviations for mean MC numbers/field for the three main effects and the skin depth are presented in Table 1. Five-way analyses of variance with two factors repeated (skin depth and distance) indicated no significant main effects for either the wound, time or special treatment conditions. However in both experiment I (Fs = 107·79, 153·89, d.f. = 1·24; the second F values refer to analyses of square roots of MC numbers/field), and experiment II

TABLE 1. Means and standard deviations (±) for mean numbers of mast cells per field for wound, time, environmental treatments and skin depth

Experiment I			Experiment II	
		Wound		
Control	Wound		Control	Wound
12·4 ± 4·5	10·9 ± 2·8		4·4 ± 1·1	3·9 ± 1·2
		Time		
2 Day	10 Day		2 Day	10 Day
11·5 ± 4·6	11·8 ± 2·9		4·2 ± 0·9	4·2 ± 1·4
		Environment		
H_2O	Acidic		Warm	Cold
11·6 ± 3·5	11·7 ± 4·2		3·8 ± 0·8	4·5 ± 1·4
		Skin depth		
Upper skin	Lower skin		Upper skin	Lower skin
7·1 ± 2·7*	16·2 ± 5·8†		2·4 ± 1·2*	6·0 ± 1·7†

* Versus † = $P < 0·001$.

(Fs = 125·91, 158·80, d.f. = 1·23) the upper skin had significantly ($P < 0·001$) fewer MCs/field than the lower tissue.

Both experiments displayed significant ($P < 0·01$) distance by wound interactions (Fs = 7·44, 14·56, d.f. = 4·96 for experiment I and Fs = 3·97, 6·45, d.f. = 4·92 for experiment II). As can be seen in Fig. 1, this interaction was due to the smaller number of MCs in the vicinity of the wound site. Interestingly, both experiments showed significant ($P < 0·05$) depth by distance interactions (Fs = 4·26, 3·00, d.f. = 4·96 for experiment I and Fs = 3·03, 6·56, d.f. = 4·92 for experiment II). *A posteriori* analyses indicated this effect was due primarily to the wounded animals (Fig. 2).

Significant wound by time (Fs = 7·05, 11·00, d.f. = 1·24), distance by wound by time (Fs = 3·12, 8·34, d.f. = 4·96), depth by distance by time (Fs = 2·14, 4·02, d.f. = 4·96) and a depth by distance by application (Fs = 3·80, 2·56, d.f. = 4·96) were also apparent in experiment I only. The first two interactions can be surmised from Fig. 3 and are due primarily to the return of MC numbers in the wound region to control values for the 10-day but not the 2-day rats. The only significant modification of MC numbers by environmental treatments, in this case, the application of either tap water or weak sulphuric acid to the wound is shown in Fig. 4. Experiment II only showed a marginally significant ($P = 0·05$) depth by wound interaction (Fs = 3·32, 5·87, d.f. = 1·23). This was due primarily to the smaller number of cells in the upper skin only (see Fig. 2) of the wounded animals relative to the non-wounded animals.

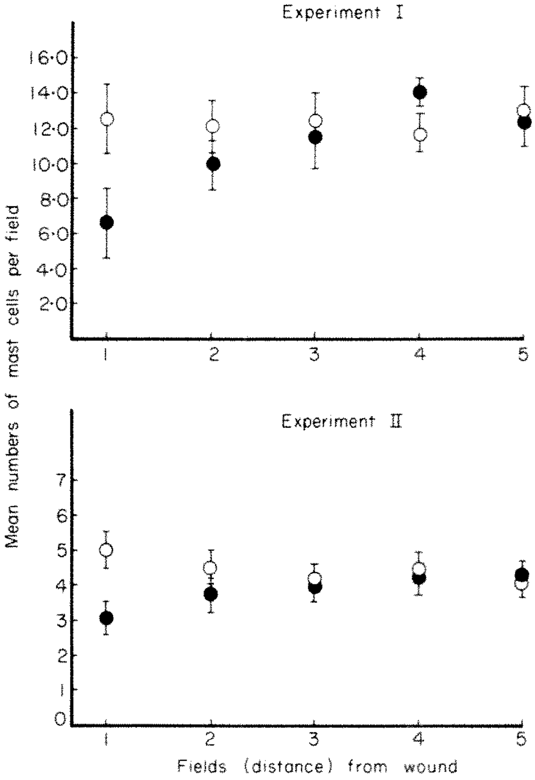

FIGURE 1. Mean numbers of mast cells/field for wounded (●) and non-wounded (○) skin as a function of the distance from the wound (or comparable reference point) for experiments I and II. Vertical bars indicate standard errors of the mean.

Analyses of co-variance for body weight, changes in body weight during the experiment or position in the housing racks did not alter the F values or produce any significant coefficients. Co-variance for thickness of the sections (for experiment I only) did not alter the F values in any significant manner.

However, there were significant ($P < 0.001$) differences in section thickness between the two kill times. Three-way analyses of variance (time by wound by treatment) demonstrated (F = 17·01, d.f. = 1·24) that the skins of the animals killed at 10 days (1·0 ± 0·2 mm) were thicker than the animals killed at 2 days (0·8 ± 0·2 mm). However, there were no significant wound or treatment effects or interactions (all Fs $\leq$ 1·00). The means and standard deviations for section thickness of the no-wound 2 day, wound 2 day, no-wound 10 day and wound 10 day groups were 0·7 ± 0·1, 0·9 ± 0·2, 1·1 ± 0·2, and 1·0 ± 0·2 mm, respectively.

Qualitative evaluation of the wound region at × 400 demonstrated features within the normal range of healing. For example, in experiment I only, three of the eight wounded rats killed after 2 days had failed to complete epithelialization; all wounded rats killed after 10 days displayed this feature. Pinkish lines and networks were noted in the fuchsin-stained sections for the 2 day wounded rats but not in the 10 day wounded rats. More material around the wound was noted by the collagen stain in the 10 day than in the 2 day tissues.

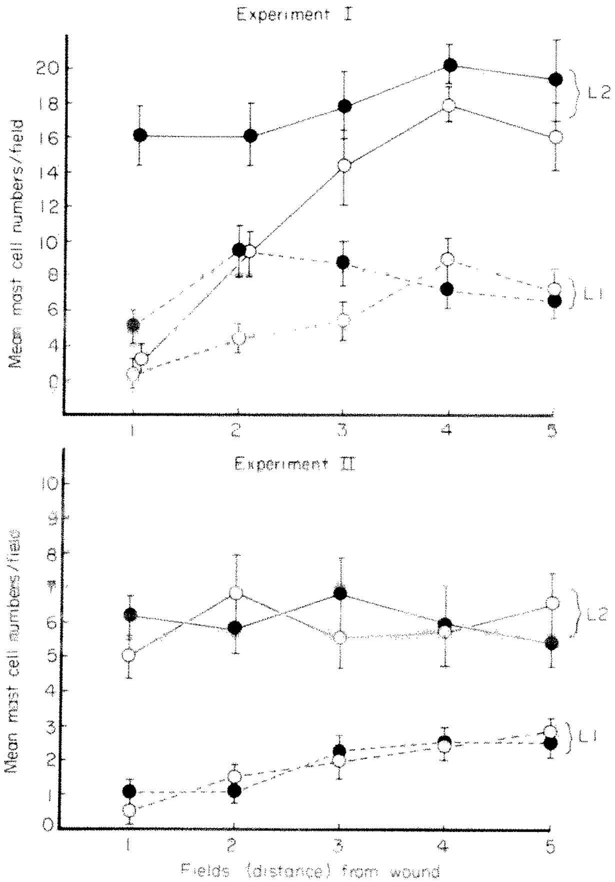

FIGURE 2. Mean numbers of mast cells/field in the upper (– – – –) and lower (———) portions of the skin as a function of distance from the wound at either 2 (○) or 10 (●) days after the incision for wounded rats only. Vertical bars indicate standard errors of the mean.

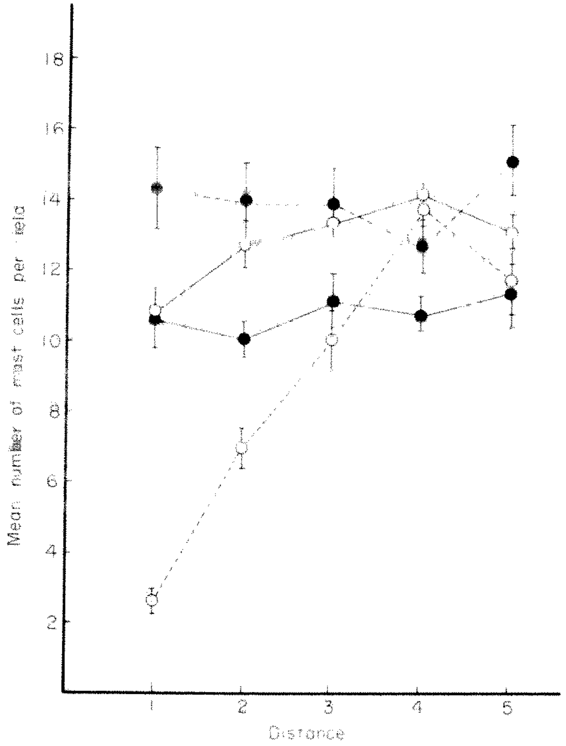

FIGURE 3. Mean numbers of mast cells/field as a function of distance for the non-wounded and wound groups on day 2 or day 10. Vertical bars are standard errors of the mean. Wound day 2 (○– – –○; wound day 10 (○———○); non-wound day 2 (●– – –●); non-wound day 10 (●———●).

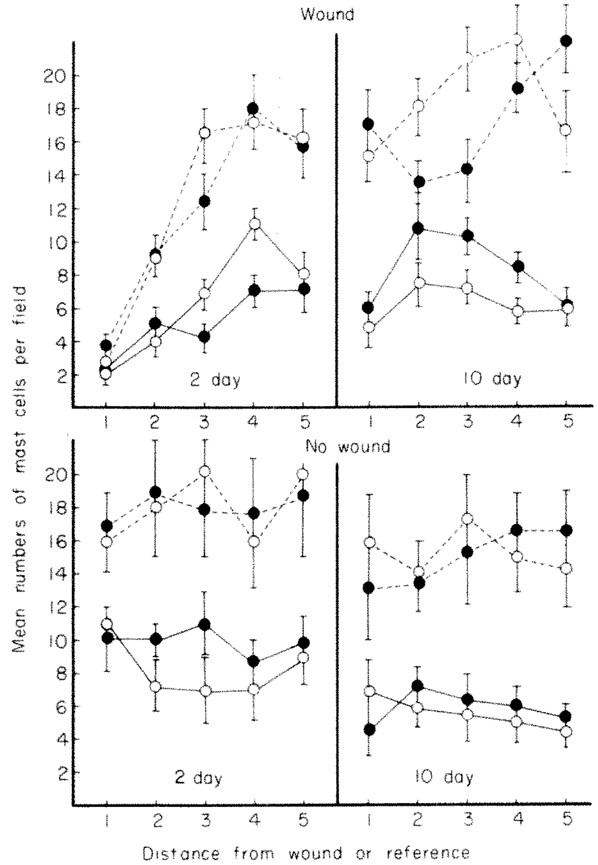

FIGURE 4. Mean numbers of mast cells/field in the upper (———) and lower (– – – –) levels of the skin for the 2 and 10 day animals as a function of distance from the wound or reference site and tap water (pH = 6·4) or weak sulphuric acid (pH = 3·5) treatments. Vertical bars indicate standard errors of the mean. pH = 6·4 (○); pH = 3·7 (●).

DISCUSSION

The present studies confirm several previous reports. Firstly, as noted by both Holczinger & Devenyi (1954) and Wilhelm, Yong & Watkins (1978), clear layer differences in MC numbers were evident. In both experiments I and II, the lower level, comparable with the dermis and hypodermis of the rat, contained about twice as many mast cells as the upper level. Estimates of mean cell density in this study ranged from 5000 to 10,000 MCs/mm^3; Wilhelm *et al.* (1978) reported a mean of about 7000 MCs/mm^3 in the skin of 60–80 day old rats.

Secondly, both the distance and time of MC changes support previous investigations. Skin MC numbers were very low (even zero in some cases) within 240 μm (radius of the microscopic field) 2 days after the wound but had returned to control group values by day 10. Boyd & Smith (1959) also noted an absence of MCs within 300 μm of the wound after 5 days of healing while Holczinger & Devenyi (1954) and Wichmann (1955) reported that the initial decrease in MCs near the wound was followed by an increase that peaked about 8–10 days later.

Distance from the wound is clearly important with respect to MC numbers. In both experiments I and II, the greatest decrease in MC numbers occurred within the first two fields or (240 + 480 μm) 0·72 mm from the wound. No obvious differences in MC numbers existed beyond this distance, at least up to five fields or 2·1 mm. However since in experiment I the 10-day-old wounded skin still had fewer MCs than non-wounded controls, low-level decrements in MC numbers may be apparent only if still greater distances from the wound are evaluated.

The wounded regions in the present studies are comparable with previous reports. For example, the pinkish network noted by the fuchsin stains had been described by Ordman & Gilman (1966) for rat skin 2 days after wounding. This line, which has been described as a collagen precursor, was not noted in the 10 day rats. Since collagen synthesis is assumed to begin about day 4-5 after wounding (Dunphy, 1974) the collagen synthesis would be expected to be well into the proliferative stage in these animals.

Certainly the major discrepancy between the two experiments was the mean MC numbers/field. Although contributions from the application of fluids to the skin and changes with age cannot be totally excluded, the most likely major source of variance was inter-observer (criterion) differences. Whereas MC counts in experiment I involved any intact metachromatic cell with the appropriate form, the MC counts in experiment II required the presence of all or a portion of the cell nucleus. Such a criterion difference would have resulted in smaller numbers of MC counts in the second experiment; the results support this suggestion. Inter-experimenter reliability measures were not completed in this study; however (Cronbach) alpha coefficients for comparable MC measurements in other experiments have been shown to exceed 0·98.

The relatively small effects of pH treatment upon MC numbers and distributions within wounded tissues were not surprising considering the short duration of the treatment application. However, the lack of significant effect from prolonged cold room exposure was surprising. If lower temperatures retard wound healing and if wound healing is related to MC distributions, then certainly some deviation should have been noted.

Our contention that central effects generated by the cold exposure would contribute to MC differences around the wound was not supported. Perhaps more peripheral factors contribute to MC density. One possibility compatible with both the MC and wound healing literature would involve the local temperature evaluation around wounded tissue (Cuthbertson & Tilstone, 1966). The facilitation of wound healing in animals exposed to higher ambient temperatures could be related to the enhanced blood flow capillary invasion around the wounded area. Considering the frequent correlation between MC numbers and capillarization, MC numbers would be expected to proliferate around wounds of animals exposed to local elevations in temperature.

Mast cells in wounded skin appear to be very mobile. Although gross enumeration of MCs and simple t-test analyses have given first approximations of MC changes around wound sites, more sophisticated statistical and analytical techniques may be required to ascertain the dynamic profiles of these cells within the skin. Multiple analyses of variance designs employing several time intervals and repeated measures (as a function of wound distance) may be helpful tools. As demonstrated, these methods can also detect weak but potentially significant alterations in MC numbers from environmental treatments.

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The role of trauma in the spreading weals of hereditary angio-oedema

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SUMMARY

Twenty patients with hereditary angio-oedema (HAO) were regularly observed. Trauma often initiates the swellings of HAO which, once formed, may continue to spread. In the prodromal rash too, a narrow band of weals spreads out from a central point, which often corresponds to a site of trauma e.g. a pin prick. The relationship of these observations to the role of trauma in the initiation and spread of weals in other forms of urticaria is discussed.

In hereditary angio-oedema (HAO) the swellings which develop may spread to surrounding tissues and this gradual extension, e.g. up an arm or across the face, is not mentioned in descriptions of this condition.

The so-called prodromal rash has been described as a reticulate erythema or erythema marginatum (Starr & Brasher, 1974; Williamson 1979; Tappeiner, Hinter & Wolff, 1980). However careful observation reveals that these are expanding rings of a narrow border of urticarial weal which then flattens and fades. It is well recognized that swellings in HAO may arise at the site of trauma, but it appears that localized trauma, often in the form of a prick, may also be the starting point of the spreading annular weal in the prodromal rash.

The purpose of this paper is to describe these phenomena in more detail and to discuss them in relation to the spread of weals and initiating trauma in other types of urticaria.

METHODS

Twenty patients with HAO were regularly observed. Sixteen of these occurred in five separate families and four were isolated cases. In twelve patients the C1 esterase inhibitor (C1 INH) was diagnostically low (type A) and in six cases, in one family, there was a functional deficiency (type B).

Four patients, all with type A deficiency and not in the same family group, had repeated attacks of the generalized rash usually referred to as the 'prodromal' rash of HAO.

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OBSERVATIONS

(i) Prodromal rash

Patients: M.H., female age 63 (no relatives affected); B.B., male age 35 (two sons affected); M.D., male age 35 (father affected), and C.E. female age 60 (one son affected). In all four patients the prodromal rash was present 1–4 days before severe attacks of abdominal colic and angio-oedema, but minor attacks were not always associated with the prodromal rash. In patients M.H. and M.D. the prodromal rash has been an obvious feature, but in patients B.B. and C.E. it has been a minor and irregular feature.

The rash is usually widespread (mainly over the trunk) and consists of weals spreading out in an annular fashion. The weal is just palpable when spreading but not during the fading stage. The spreading edge is 2–5 mm across. Itching is not a feature. The wealed edge of the ring spreads over a period of 2–4 days and reaches a diameter of 5–15 cm before fading. This means that the edge advances at a rate of 2–3 mm/h. The spread is uneven and in some sites, e.g. on the hand, it is more apparent in one direction and an arcuate narrow weal spreads up the forearm (Fig. 1). In other sites, e.g. on the front of the elbow, a more regular ring develops. If very

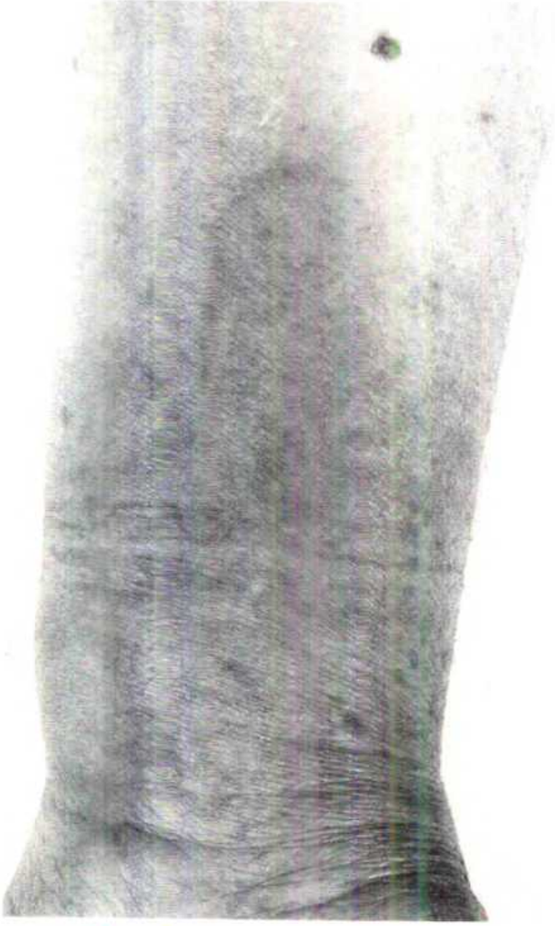

FIGURE 1. HAO prodromal rash. Edge of weal spreading up the forearm 36 h after beginning on the front of the wrist.

numerous, the spreading weals may merge into each other and produce a widespread eruption with arcuate lines.

Patients M.H. and M.D. have both observed that the centre of the expanding ring may correspond to a site of trauma. For example, both have had spreading rings developing round the site of venupuncture, first noticed on the following day. Patient M.H. has also had such rings start at the site of a sewing needle prick and at a minor puncture from a jagged finger nail and patient M.D. developed a spreading ring after a scratch from a carpenter's nail. Similar trauma at times other than during the prodromal period will not cause weals to develop. Patient M.D. also noticed that the rings may spread from and surround the site of acneform pustules on his chest and back and this was clinically obvious to the observer (Fig. 2).

(ii) Angio-oedema swellings

Once formed an angio-oedema swelling often spreads, e.g. up a limb or across the face, moving a distance of 10–20 cm in the course of 1–2 days (3–4 mm/h). A blow on the temple in patient C.E. caused a swelling of the adjacent eyelid; in 24 h this had spread across the forehead and, in a further 24 h, to the upper lip. Similarly patient M.D. received a knock on the back of the hand in

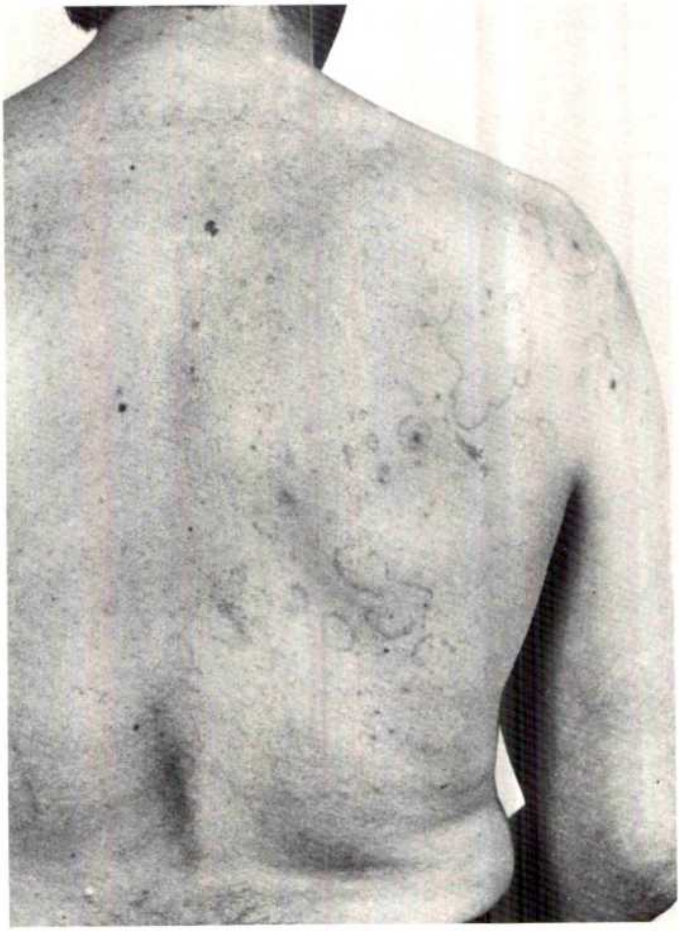

FIGURE 2. HAO prodromal rash. Acneform pustules at the centre of the expanding rings.

the morning. By evening the hand was swollen and in 24 h the swelling involved the wrist. In a further 24 h it had spread to the top of the forearm.

Although many angio-oedema swellings develop without any history of preceding trauma, thirteen out of the twenty patients reported that localized trauma had at some time started off a swelling, whereas on other occasions similar trauma had had no effect. Examples of trauma initiating weals in these patients include dental extraction, a blow from a tennis ball, a knock against a window sill, and knocks against machinery at work. Abnormal pressure will sometimes initiate the weals, e.g. one patient developed a weal in the palm of the hand following the use of nut crackers and another patient had a swollen hand after cracking walnuts against each other. One had swellings in the vulval area on the morning after intercourse. In most cases the swelling is noted some 4–12 h after the trauma but it may be on the following day.

DISCUSSION

Weals which spread, often becoming partially annular or with arcuate edges, are seen in many different types of urticaria apart from HAO including acute and chronic urticaria, serum sickness and vasculitis urticaria. This phenomenon was mentioned by Warin & Champion (1974) but the author has since made further measurements of the weal edge at regular intervals in six patients with chronic urticaria and two with vasculitis urticaria. The rate of spread varies, but it is about 3–4 mm/h. It becomes slower as the weals get older, flatten and fade. In parts of the weal edge the spread is more rapid (Figs 3a and 3b). Angio-oedema swellings may also spread, and although it is not so easy to mark the edge, a spread of 10 cm takes about 24 h.

The mechanism of this spread is not known, but in HAO a rational explanation would be that at the central point, complement changes are initiated leading to release of vasoactive substances. These or their tissue effects then lead to further complement changes and more vasoactive chemicals at the periphery but not towards the centre, as presumably various contributory substances have been used up. Perhaps a similar mechanism is responsible for the spreading weals seen in other types of urticaria.

Similarly, trauma may also initiate weals in urticaria which is not due to HAO. Pressure urticaria usually follows continued pressure, but sometimes a heavy blow will also cause a weal to develop in this condition. The weal appears some 4 h after the trauma. In ordinary chronic urticaria and angio-oedema weals may develop at the site of pressure. This usually happens at the height of the attack and the pressure is merely responsible for the positioning of the weal (Köbner phenomenon weals). The issue is complicated by the fact that about a third of the patients with undoubted pressure urticaria may also get chronic urticaria from time to time. It is therefore theoretically possible to get true pressure urticaria and Köbner phenomenon weals in the same subject. Acute serum sickness and vasculitis urticaria may also show some weals occurring at the site of pressure or trauma.

There are various possible explanations for trauma or pressure initiating weals in these different types of urticaria. In the case of pure pressure urticaria the suggestion is that trauma sets up a chain of pharmacological reactions which liberate vasoactive substances responsible for the localized oedema. In serum sickness and vasculitis urticaria it would appear likely that immune complexes are lodged at the site of pressure or trauma. In HAO because of the reduction of the C1 esterase inhibiting effect the trauma initiates the complement changes with the subsequent liberation of vasoactive substances.

It is interesting that initiation of the expanding weal in the HAO prodromal rash can occur not

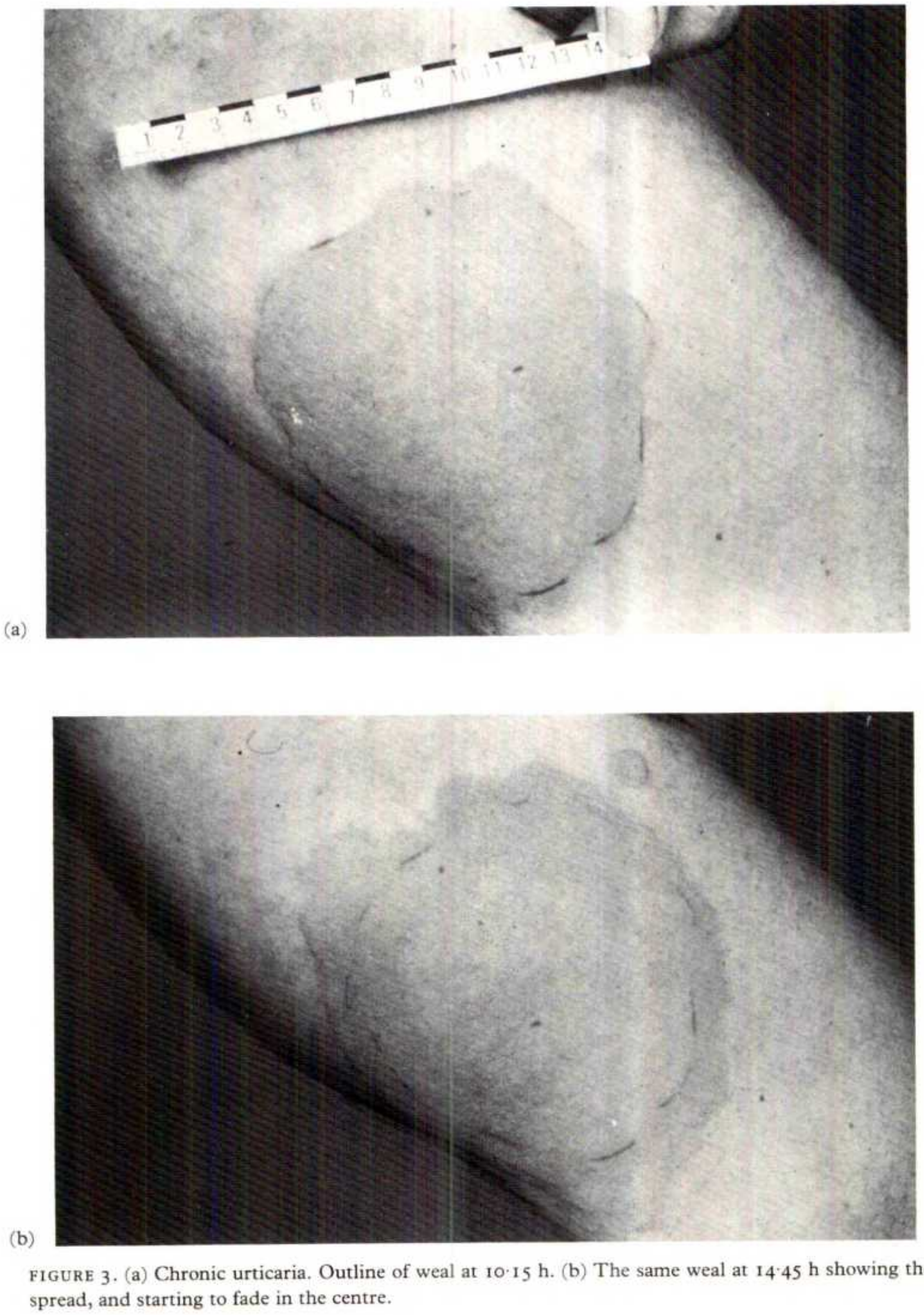

FIGURE 3. (a) Chronic urticaria. Outline of weal at 10·15 h. (b) The same weal at 14·45 h showing the spread, and starting to fade in the centre.

only from localized trauma, but also from an acneform folliculitis. Presumably the inflammation in the latter condition causes similar complement effects to those due to trauma.

In spite of these possible different mechanisms the final clinical appearance of the weals and swellings is very similar in all these different types of urticaria.

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Pharmacology and Treatment

A double-blind comparison of sulconazole nitrate 1% cream with clotrimazole 1% cream in the treatment of dermatophytoses

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SUMMARY

Forty patients with dermatophytosis were treated with either sulconazole 1% cream or clotrimazole 1% cream (twenty patients in each group) twice daily for 4 weeks.

Overall clinical improvement with respect to baseline at weeks 2, 3 and 4 favoured sulconazole-treated patients (the differences were statistically significant). The weekly mean severity scores for the sulconazole-treated patients for erythema, scaling, fissuring and itching were consistently lower than those for the clotrimazole-treated patients and the differences were statistically significant for erythema and sealing.

It was concluded that sulconazole nitrate 1% cream is very effective against various dermatophytoses.

Sulconazole nitrate (+)-1-2-(4-chlorobenzylthio-2-(2,4-dichlorophenyl)-ethyl imidazole nitrate) is an imidazole derivative synthesized by Syntex Corporation. It has been formulated in a cream for the topical treatment of fungal infections of the skin. The drug exists as tiny, emulsified globules dispersed throughout the cream, with a small amount of solubilized nitrate.

Clotrimazole is a widely used agent for the topical treatment of fungal infections of the skin. It has been shown to exert a good antifungal activity (Sawyer *et al.*, 1975) and has been shown to be clinically effective and well tolerated in the topical treatment of several conditions, for example tinea pedis (Smith, Graham & Ulrich, 1977).

We report here a double-blind parallel study of sulconazole nitrate 1% cream compared with clotrimazole 1% cream in the topical treatment of dermatophytoses in forty patients.

PATIENTS AND METHODS

A total of forty patients with dermatophytosis, verified by KOH examination and culture, twenty in each treatment group, were enrolled in the study (Table 1). The mean ages and sex distribution of the patients in the two groups did not differ significantly.

In all patients, samples from the monitored lesion were taken for KOH preparation and

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TABLE 1. Clinical data of the forty patients treated

	Sulconazole	Clotrimazole
Mean age (years)	41·2	44·0
Males	16	15
Females	4	5
Site Toe webs	15	13
Groin	5	6
Plantar surface	0	1
Organism isolated		
Trichophyton mentagrophytes	9	9
Trichophyton rubrum	7	6
Epidermophyton floccosum	3	3
Trichophyton species	1	1
Candida albicans	0	1

fungal culture before start of treatment and at each follow-up visit. The cultures were performed on Sabouraud's medium. In order to detect possible late growths the cultures were followed for up to 6 weeks.

The two test preparations were presented in tubes of identical appearance and the patients were randomly allocated to either treatment. The code was not broken until the study was complete. Informed consent was obtained from each patient before the start of treatment.

The allowable visit ranges for the follow-up visits were as follows: for the week 2 visit, study days 13–18, for the week 3 visit, days 19–26, and for the week 4 visit, days 26–34. At 6 and 12 weeks after completion of treatment, thirty-four patients who were KOH and culture negative and completely clear of all clinical signs and symptoms at the week 4 visit, returned for post-treatment visits.

The patients were instructed to treat the infected lesions twice daily. No other topical or systemic treatments were allowed during the whole study period.

The investigating physician at each visit also recorded and graded from 1 (absent) to 4 (severe) the following clinical signs and symptoms: erythema, itching, scaling, maceration, fissuring and vesiculation. An evaluation of overall clinical improvement as compared to initial visit and graded from 1 (worse) to 5 (completely clear) was also made at each visit. At the end of treatment, the patients were asked to assess the cosmetic acceptability of the preparations.

The following statistical methods were used: Fisher's exact test, Wilcoxon rank sum test and Wilcoxon signed rank test. Statistical tests were performed to test for significant changes from admission (baseline), and in order to test for significant differences between the treatment groups.

RESULTS

One patient (clotrimazole-treated) was excluded from week 4 efficacy analyses because her visit was outside the acceptable range. Two patients did not complete the treatment because of adverse reactions and were therefore excluded from all but demographic analyses. One patient did not give a culture result at week 2 and was therefore not included in culture analysis at that visit.

There were no statistically significant differences at any time between the two treatment groups with respect to KOH and culture results (Table 2), but during the first 3 weeks of

TABLE 2. Mycology results

No. of patients	Admission S	Admission C	Week 2 S	Week 2 C	Week 3 S	Week 3 C	Week 4 S	Week 4 C
KOH positive	19	19	3	6	1	3	0	1
Culture positive	19	19	1	2	0	1	0	0
Total	19	19	19	18	19	19	19	18

S = Sulconazole. C = Clotrimazole.

treatment, the number of positive cultures was consistently lower in the sulconazole-treated group.

Erythema, scaling, itching and fissuring all responded better to sulconazole than to clotrimazole. For erythema and scaling, a statistically significant difference in favour of sulconazole was observed at weeks 2, 3 and 4 of treatment (Table 3). All patients in whom vesiculation was noted at baseline were free of this symptom after 2 weeks treatment. The mean severity of maceration scores for the sulconazole-treated patients at weeks 2, 3 and 4 were consistently lower than corresponding values for clotrimazole-treated patients. However, the severity of maceration at baseline was significantly greater in the clotrimazole treated group.

Overall clinical improvement with respect to baseline at weeks 2, 3 and 4 favoured sulconazole-treated patients ($P < 0·05$ at all visits). A 'significant' or 'completely clear' improvement was experienced by 94·8, 100·0 and 100·0% of sulconazole-treated patients at weeks 2, 3 and 4, respectively. Corresponding weekly improvements with clotrimazole were 73·7, 78·9 and 83·4%.

TABLE 3. Mean severity responses for clinical symptoms (1 = absent, 2 = mild, 3 = moderate, 4 = severe)

	Admission S	Admission C	Week 2 S	Week 2 C	Week 3 S	Week 3 C	Week 4 S	Week 4 C
Erythema	3·3	3·1	2·3	2·5	1·3	1·8	1·0	1·4
P-value*	NS		0.05		0.01		0·05	
Scaling	3·2	3·2	1·9	2·4	1·2	1·9	1·0	1·4
P-value*	NS		0·05		0·01		0·05	
Itching	3·4	3·5	1·7	2·1	1·0	1·6	1·0	1·4
P-value*	NS		NS		NS		NS	
Fissuring	2·6	3·0	1·3	1·7	1·0	1·5	1·0	1·5
P-value*	NS		NS		NS		NS	
Vesiculation	2·6	2·6	1·0	1·0	1·0	1·0	1·0	1·0
P-value*	NS		NS		NS		NS	
Maceration	2·6	3·4	1·1	2·3	1·0	2·0	1·0	2·0
P-value*	0·05		NS		NS		NS	

* Wilcoxon rank sum test using differences from admission at each follow-up visit. NS = Not significant.
S = Sulconazole. C = Clotrimazole

Tinea pedis

Since the majority of the patients had tinea pedis, this group was analysed separately. As with the total group of patients, the improvement in erythema and scaling was significantly greater in the sulconazole treated group. For overall clinical improvement, statistically significant differences ($P < 0{\cdot}05$) favouring sulconazole were found at weeks 2, 3 and 4.

The overall investigator efficacy rating for tinea pedis patients was 'excellent' for all cases treated with sulconazole. Three of the clotrimazole treated patients, however, received poor ratings: scores were 'excellent' for nine and 'fair' for one.

Post-treatment follow-up

At 6 and 12 weeks after completion of the treatment (week 10 and 16, respectively) thirty-four patients, who had negative KOH and culture and were clinically clear of fungal infection, returned for post-treatment follow-up. Two clotrimazole patients but none of the sulconazole patients had a relapse. This difference is not statistically significant using the Fisher's exact test. The thirty-two patients not experiencing a relapse at week 10 continued through to week 16 without any sign of relapse.

Adverse reactions

One patient from each treatment group experienced an allergic reaction to the test preparation during the first week of treatment. The treatment was therefore discontinued in both cases. In the sulconazole-treated patient the reaction was confirmed by patch testing with the test medication (+ + +). No other adverse reactions were observed. Both tested creams were cosmetically well accepted by all patients.

DISCUSSION

Sulconazole proved to be more effective than clotrimazole in the topical treatment of various dermatophytoses. This was observed throughout the whole treatment period of 4 weeks in both the clinical and the mycology studies. The long post-treatment follow-up period was performed in order to detect possible latent infections causing recurrences after the treatment. No patients in the sulconazole-treated group experienced a late recurrence, but this occurred in two patients in the clotrimazole-treated group. The difference was not related to the organism causing the infection. One patient in each group experienced an allergic reaction to the test preparation. In the sulconazole-treated patient this must have been due to a cross-reaction with either another imidazole derivative or another vehicle component, because the reaction occurred after only 4 days of treatment.

The present study has shown that sulconazole 1% cream is very effective against various dermatophytoses, and it will be a valuable alternative to currently available topical therapies.

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Benzoyl peroxide versus topical erythromycin in the treatment of acne vulgaris

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SUMMARY

In a double-blind clinical study in ninety-four subjects a 1·5% (w/v) erythromycin lotion was as effective as 5% (w/v) benzoyl peroxide gel in significantly reducing the number of small inflamed lesions and the overall acne severity. However, benzoyl peroxide also significantly reduced the number of non-inflamed lesions whereas erythromycin had no effect on these lesions. This study supports the view that, although topical erythromycin is of value in the treatment of mild or moderate acne vulgaris, long established, safe and effective remedies should not be replaced by topical antibiotics until more comparative studies and investigations on bacterial resistance have been completed.

Although oral antibiotics continue to be the mainstay of current acne therapy, the last decade had seen the introduction of more effective topical remedies including vitamin A acid and benzoyl peroxide. The latter substance is available in a wide variety of preparations and has proved useful in the therapy of mild acne and as an adjunct in the management of more severe cases. The efficacy of benzoyl peroxide has been demonstrated by many authors (Kligman, Fulton & Plewig, 1969; Fulton, Farzad-Bakshandeh & Brackey, 1974; Cunliffe, Dodman & Ead, 1978; Cunliffe, Burke & Dodman, 1980), as has its ability to reduce bacterial numbers and the percentage of free fatty acids in surface sebum (Fulton *et al.*, 1974; Leyden *et al.*, 1980; Cunliffe & Holland, 1981). The use of benzoyl peroxide, in the majority of patients, is not associated with any side-effects, apart from mild irritation during the first few days of application and it is rarely contra-indicated.

Since the mid-seventies there has been a steadily increasing use of topical antibiotic preparations for the treatment of acne and currently six commercial formulations are available in the U.S.A. There are already over fifty publications on the use of topical preparations of clindamycin, tetracycline and erythromycin in acne therapy (Eady, Holland & Cunliffe, 1982). Most of these claim efficacy for the preparation tested and minimal side-effects. Little consideration has been given to the risks of promoting bacterial resistance in the treated

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population and in their contacts. At present there is widespread concern over the indiscriminate use of antibiotics, and limitations on practices which promote bacterial resistance are being sought (Marshall, 1981). Topical antibiotics are already widely prescribed in the U.S.A. without proof that their use is not associated with the promotion of bacterial antibiotic resistance.

Comparative studies between oral and topical antibiotics are few (Robinson, 1954; Blaney & Cook, 1974; Anderson, Cook & Smith, 1976; Smith, Chalker & Wehr, 1976; Stoughton *et al.*, 1980) but it is likely that oral antibiotics are more effective. Similarly comparisons between topical antibiotics are lacking so that the most effective one is not certainly known. Surprisingly, especially because of the potential hazards of topical antibiotic use, no published study has compared benzoyl peroxide with topical antibiotics in the treatment of acne. Such a study is obviously essential to the dermatologist to help him choose the preparations which should be used topically for patients with acne. This paper reports the findings of a double-blind clinical trial of 1·5% (w/v) erythromycin versus 5% (w/v) benzoyl peroxide.

METHODS

This study was a double-blind comparative group assessment of three test formulae: 5% (w/v) benzoyl peroxide in a gel base, 1·5% (w/v) erythromycin and the erythromycin base which contained SP alcohol 40 0·6 ml/ml (equivalent to 0·555 ml ethanol), propylene glycol 0·36 ml/ml, Laureth 4 60·0 mg/ml, perfume (W1942-1) 1 mg/ml. For logistic reasons the benzoyl peroxide base could not be included in the study.

Ninety-four subjects with mild or moderate acne were recruited from the local student population, all but five of whom completed the trial. Table 1 shows the relevant clinical details. At each visit the acne was graded on a subjective scoring system which involved both inspection and palpation of the skin (Cunliffe, 1981). Spot counts were made of the blackheads and whiteheads (total non-inflamed lesions—TNIL) and of the pustules and papules (total small inflamed lesions—TIL). Macules, which probably represent resolving inflamed lesions, were also counted.

On the first visit the subjects were divided into three groups matched as far as possible for age, sex, acne grade and the number of superficial inflamed and non-inflamed lesions. They were asked to apply the selected preparation twice daily and were assessed at the initial pretreatment visit and after 4 and 8 weeks of therapy.

RESULTS

Table 2 shows the results of lesion counts obtained at weeks 0, 4 and 8. Within-group changes in

TABLE 1. Details of patients studied

	Placebo	Erythromycin	Benzoyl peroxide
Number of patients	29	30	30
Sex: male	23	25	22
female	6	5	8
Age range (years)	18–20	18–20	18–20
Mean age (years)	18·8	18·8	19·0

TABLE 2. Summary of lesion count data at weeks 0, 4 and 8 (mean ± s.e.m.)

Type of lesion	Time of count (weeks)	Placebo ($n = 29$)		Erythromycin ($n = 30$)		Benzoyl peroxide ($n = 30$)		Between groups
		Mean	s.e.	Mean	s.e.	Mean	s.e.	
Total (non-inflamed)	0	32·4	3·6	33·3	3·8	32·8	3·1	
	4	30·7	2·5	30·6§	3·8	24·9‡	2·6	BP > E*
	8	31·6	3·0	29·9	3·2	23·2¶	2·8	BP > E† BP > P†
Total	0	17·5	2·0	21·3	3·3	19·3	2·5	
Total (small inflamed)	4	18·6	2·6	12·4‡	1·6	12·9†	1·9	BP > P† E > P†
	8	16·6	2·6	13·1¶	1·4	12·2	1·8	BP > P† E > P†
Macules	0	8·5	0·9	7·5	0·9	10·3	1·5	
	4	11·2	0·9	9·6	1·0	10·2	1·3	
	8	10·3	0·9	7·8	1·2	8·0†	1·5	BP > E*
Total lesions	0	59·3	5·2	60·9	6·6	62·7	5·4	
	4	60·6	4·1	51·3†	5·3	47·8‡	4·6	BP > P‡ E > P†
	8	59·4	4·1	48·9†	5·0	43·3‡	4·6	BP > P‡ E > P†

*†‡ Difference between groups significant at 5%, 1% and 0·1% levels, respectively.
§¶ Difference between groups significant at 2% and 0·2% levels, respectively.
P, placebo.
E, erythromycin.
BP, benzoyl peroxide.

lesion count from week 0 were evaluated using a paired t-test. Between-group comparisons were performed using analysis of covariance with the week 0 count as covariate.

Total non-inflamed lesions

Benzoyl peroxide treatment produced a significant reduction in TNIL at 4 (24%, $P < 0{\cdot}001$) and 8 weeks (29%, $P < 0{\cdot}002$). The erythromycin-treated group showed a slight but significant reduction after 4 weeks (8%, $P < 0{\cdot}02$) but not after 8 weeks. There was no change in the TNIL counts of the group treated with the erythromycin base. A between-group difference in favour of benzoyl peroxide was found at weeks 4 ($P < 0{\cdot}05$) and 8 ($P < 0{\cdot}01$) in comparison with erythromycin.

Total small inflamed lesions

Both benzoyl peroxide and erythromycin produced a reduction in TIL at the fourth and eighth weeks (the benzoyl peroxide by 33% and 37% and the erythromycin by 42% and 39%). These reductions were significant at the following levels: benzoyl peroxide $P < 0{\cdot}01$ and erythromycin $P < 0{\cdot}001$, respectively. There was no reduction with the erythromycin base.

Between-group comparisons showed that both active preparations were significantly better than the base at the first and second month ($P < 0{\cdot}01$ in each case). There was no significant difference between the benzoyl peroxide and erythromycin preparations.

Macules

The only significant change in macule count was a decrease shown by the benzoyl peroxide-treated group at week 8 (22%, $P < 0{\cdot}01$). There was no reduction in the erythromycin-treated group.

TABLE 3. Grade and effect of treatment

Time of count (weeks)	Placebo ($n = 29$)		Erythromycin ($n = 30$)		Benzoyl peroxide ($n = 30$)	
	Mean	s.e.	Mean	s.e.	Mean	s.e.
0	0·57	0·06	0·72	0·09	0·70	0·09
4	0·68	0·07	0·59*	0·06	0·56*	0·05
8	0·62	0·06	0·50†	0·05	0·42†	0·06

*† Significant at the 1% and 0·1% levels of significance as measured by Wilcoxon test.

Clinical grading

The clinical grading scores assigned by the investigator are summarized in Table 3. Both erythromycin and benzoyl peroxide significantly improved the grade at 4 and 8 weeks (erythromycin by 19% and 32%, benzoyl peroxide by 19% and 39%). These changes were significant at 4 and 8 weeks for both preparations ($P < 0·05$ and $P < 0·01$, respectively). The changes in grading were evaluated by the Wilcoxon matched pairs test. No between-group differences were found when changes in grade were compared by the paired Mann-Witney test.

Side-effects

Mild erythema and scaling were observed in only a small proportion of cases. Two patients developed clinical dermatitis, one in the erythromycin and one in the benzoyl peroxide-treated group. They presented with the dermatitis 10–20 days after starting therapy and had to withdraw from the study. Patch tests to determine whether these were instances of allergic contact dermatitis have not been carried out.

DISCUSSION

The results of this study confirm the clinical efficacy of benzoyl peroxide and topical erythromycin and show that both treatments were equally effective in reducing the number of superficial inflamed lesions. This is not surprising since both agents are antimicrobial and, in addition, erythromycin has been shown to possess anti-inflammatory properties (Plewig & Schöpf, 1975). Benzoyl peroxide had a much greater effect than erythromycin on the number of non-inflamed lesions. This might be explained by the 'comedolytic' effect of benzoyl peroxide which has been well documented (Kaidbey & Kligman, 1975; Kligman *et al.*, 1975).

The original study on topical erythromycin by Fulton & Pablo (1974) demonstrated a 39% reduction in non-inflamed lesions after 8 weeks therapy with 2% (w/v) erythromycin. Feucht *et al.* (1980) found a significant reduction in non-inflamed lesions with topical 4% (w/v) erythromycin/zinc liquid after 10 weeks, but not with 4% (w/v) erythromycin/zinc gel. Bernstein & Shalita (1980a) found no reduction in comedone count after 8 weeks therapy with 2% (w/v) erythromycin. Therefore, it is still not yet clear whether topical erythromycin alone has any effect on non-inflamed lesions or whether the reduction recorded by some authors is due to other factors such as composition of the vehicle. It is interesting to note that there are no reports of a reduction in non-inflamed lesions with oral erythromycin.

The erythromycin base used in this study exerted no effect on the inflamed or non-inflamed lesions. This contrasts with the findings of Dobson & Belknap (1980), who used the same base. The degree of response to the active preparation, as assessed by reduction in the number of inflamed lesions, was also greater in the latter study. Both discrepancies might be explained by the fact that the trial reported by Dobson & Belknap (1980) was a multi-centre study, in which assessment of degree of improvement would vary between different investigators.

The effects of extensive topical use of erythromycin on the emergence of resistance in the skin microflora have been largely ignored. However, an increase in the percentage of erythromycin resistant staphylococci on the face after topical therapy has already been reported (Mills, Kligman & Stewart, 1975), as has an increase in the number of individuals with erythromycin-resistant organisms (Bernstein & Shalita, 1980b). Resistance in propionibacteria following topical use of erythromycin has also been reported (Crawford *et al.*, 1979). This resistance was, in every case, linked to clindamycin resistance. Despite many years of oral use in acne therapy resistant propionibacteria did not emerge. Another possibility is the transfer of active antibiotic from the skin of the treated individual to untreated contacts in levels high enough to promote resistance in their microflora. Levels of antibiotic on the skin surface following topical use are over 1000 times higher than those obtained during oral use (Eady *et al.*, 1982).

It is worth remembering the numerous outbreaks of serious infection due to multiple resistant *Staphylococcus aureus* which occurred following extensive use of topical gentamicin (*Lancet* editorial, 1981).

We do not suggest that topical antibiotics for the treatment of acne should be withheld but detailed studies must be made in several centres to investigate the possibility of increased bacterial antibiotic resistance in the community occurring as a result of widespread use of topical antibiotics for acne therapy. This study has shown that 5% (w/v) benzoyl peroxide is at least as effective as 1·5% (w/v) erythromycin. Dermatologists should not be tempted to discard a safe, successful topical treatment for acne until the newer therapies have been proved equally safe and effective.

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Case Reports

Secondary amyloidosis affecting the skin in arthropathic psoriasis

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SUMMARY

A case of secondary systemic amyloidosis associated with arthropathic psoriasis is reported. Amyloid A protein was demonstrated in the kidneys, gastrointestinal tract and liver, and also in psoriatic and clinically uninvolved skin.

CASE REPORT

J.G., now aged 33 years, first attended Glasgow Royal Infirmary in 1960 with plaque psoriasis of scalp and limbs, present since the age of 7 years. Plaques gradually increased in number and size and he has never been completely clear of psoriasis. He developed arthritis of the left ankle in 1964 and by 1972 widespread arthritis had developed involving both ankles, knees, wrists, left shoulder, fingers, toes, lumbar spine and right sacroiliac joint. His skin biopsy at that stage was consistent with psoriasis vulgaris. Rheumatoid factor was not demonstrable and radiological examination of involved joints showed typical psoriatic arthropathic changes. By 1975, he had developed fairly widespread psoriasis with extensive mutilating deformities of his wrists and finger joints, requiring repeated surgical treatment with arthrodesis. In January 1979, he developed haematuria and a markedly raised alkaline phosphatase of 2047 U/l (normal 80–280 U/l); detailed renal and gastrointestinal investigations were carried out, but no abnormalities were found. Liver biopsy showed mild reactive changes and jejunal biopsy was essentially normal. Isoenzyme inactivation studies on alkaline phosphatase confirmed it to be of hepatic origin. Intravenous pyelography suggested the possibility of renal infarct but his haematuria cleared before renal arteriography could be carried out and it was concluded that he had developed analgesic nephropathy. There is a strong family history of psoriasis with his mother, younger sister and his eldest daughter, now aged 10 years, having the disease but none of them have arthropathy.

The mainstay of his treatment over the years has been varying concentrations of coal tar or dithranol with short courses of topical steroids. He has required in-patient treatment for acute exacerbations of his psoriasis at least once every year. In addition, he had trials with intravenous

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methotrexate for 4 months in 1971, hydroxyurea for 3 months in 1972 and azathioprine for 2 months in 1978, but each trial had to be stopped because of side-effects. In addition, he has taken a variety of analgesics and anti-inflammatory agents for his joints over the years, including indomethacin, dihydrocodeine, hydroxychloroquine, sulindac, ibuprofen, peroxicam, fenprofen and a trial of prednisolone 15 mg daily for 1 year in 1975.

The severity of his psoriasis and failure to respond to conventional therapy warranted the use of methotrexate again in March 1980, which he tolerated well and his psoriasis showed good response on the doses of 10–20 mg intravenously every week on which he is continuing.

In July 1980, he again developed an acute exacerbation of psoriasis together with massive proteinuria and he was admitted for investigation. Examination at this stage revealed a normal blood pressure and no peripheral oedema. There was no hepatic, splenic or lymph node enlargement and his tongue was normal. He had extensive bilaterally symmetrical psoriatic plaques on limbs, trunk and scalp and on the dorsal aspects of hands and feet with gross nail changes. In addition, he had mutilating arthropathic deformities of wrists, fingers, toes and active arthropathy of right wrist, left shoulder and both ankles.

INVESTIGATIONS

In July 1980 he had a marked proteinuria of 4·62 g/l. Serum urea was 4·6 mmol/l, serum creatinine 75 μmol/l and creatinine clearance 134 ml/min. His midstream specimen of urine was negative for white or red blood cells and casts and was sterile on three consecutive cultures. Culture of urine for acid fast bacilli was also negative. No Bence-Jones proteins were detected. His haemoglobin was 13·0 g/dl with a high ESR of 100 mm in the first hour. Cell counts and cell morphology were within normal limits. Total serum proteins were 63 g/l with a low albumin of 25 g/l and a globulin level of 38 g/l. Serum protein electrophoresis revealed raised α_1 and α_2 globulins. Alkaline phosphatase was 736 U/l.

Other investigations yielding normal or negative results included serum calcium, phosphate, uric acid, complement screen, cryoglobulins, immunoglobulins, rheumatoid factor, antinuclear factor, LE latex slide test, VDRL, virology screens (including hepatitis B antigen), Ba meal, oral cholecystogram and liver and spleen scans.

Intravenous pyelography revealed papillary necrosis in both kidneys suggesting analgesic nephropathy.

HISTOPATHOLOGY

Biopsy of the left kidney yielded sixteen glomeruli for study. Sections when stained with Sirius red stain revealed amyloid in afferent arterioles, glomerular capillary loops and interstitial vessels. Polarization microscopy of these sections gave the anomalous green birefringence typical of amyloid. Sections from psoriatic skin and the clinically uninvolved skin (Fig. 1) revealed amyloid deposits in the dermal papillae when stained with Sirius red and both sections revealed green birefringence of amyloid deposits. The potassium permanganate reaction of Wright, Calkins & Humphrey (1977) was performed on sections from kidney, psoriatic and clinically uninvolved skin which made the amyloid lose its staining and birefringence, proving it to be an amyloid of protein AA type.

Immunoperoxidase studies on sections from psoriatic skin, clinically uninvolved skin and left kidney were performed using the peroxidase-antiperoxidase technique of Sternberger *et al.* (1970) with anti-human tissue A component (Atlantic Antibodies) supplied by American Hospital Supplies (U.K.) Ltd. This could be almost completely absorbed with serum from a

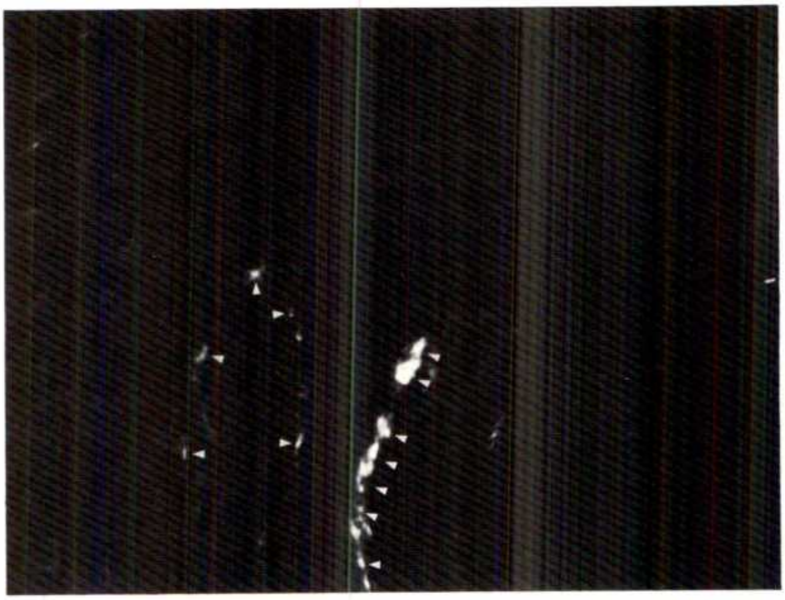

FIGURE 1. Polarization microscopy of Sirius red stained sections showing anomalous green birefringence of amyloid deposits (arrows) in dermal papilla in clinically uninvolved skin (× 158).

known case of amyloidosis secondary to tuberculosis (SAA) and was further controlled by simultaneous incubation of sections with normal goat serum, yielding no staining of amyloid. This test gave clear staining of amyloid in sections from kidney, clinically uninvolved and psoriatic skin (Fig. 2).

A review of the patient's liver biopsy from 1979 and his jejunal biopsy from 1980 also gave

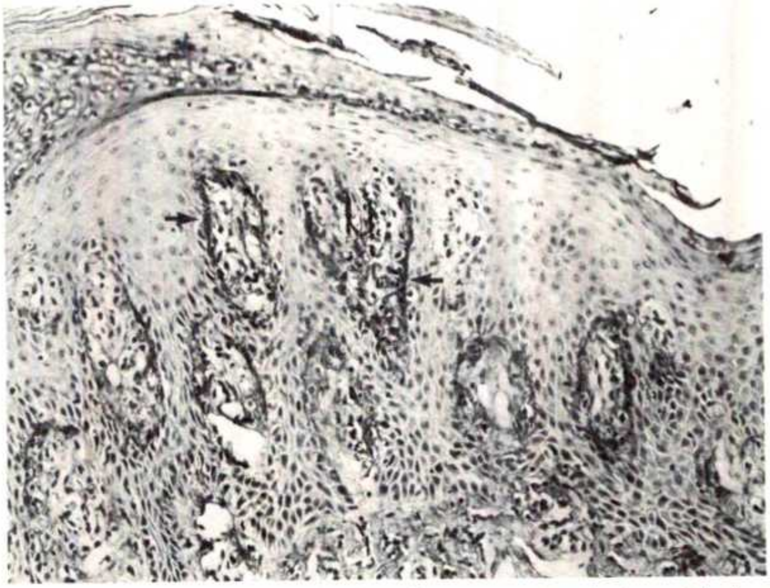

FIGURE 2. Immunoperoxidase with anti-human tissue A component deposits (arrows) in dermal papillae in psoriatic skin (× 158).

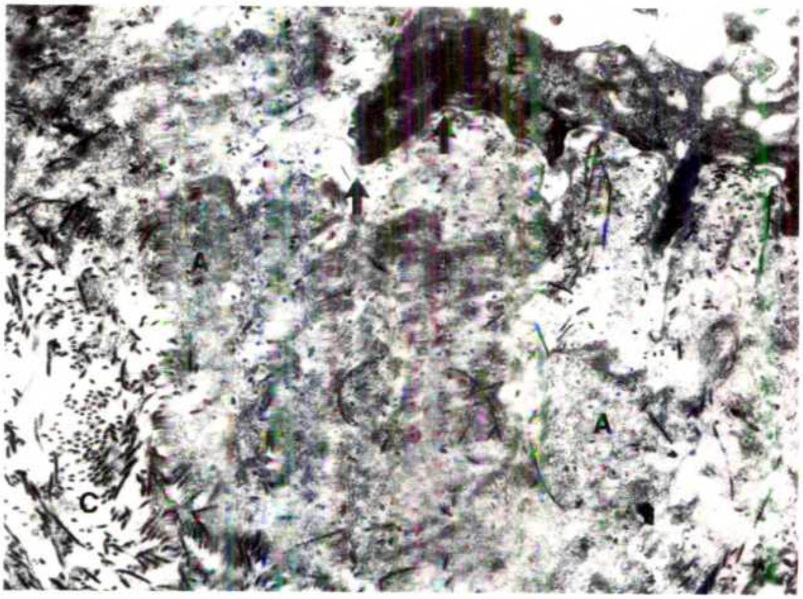

FIGURE 3. Electron micrograph of clinically uninvolved skin showing amyloid fibrils (A) beneath epidermal basement membrane (arrows) and superficial to the dermal collagen fibrils (C) with part of an epidermal cell (E) included for orientation (× 9,280).

positive staining with Sirius red in the blood vessels with a positive green birefringence by polarization microscopy.

Electron microscopy was performed on glutaraldehyde fixed epon-embedded tissue from

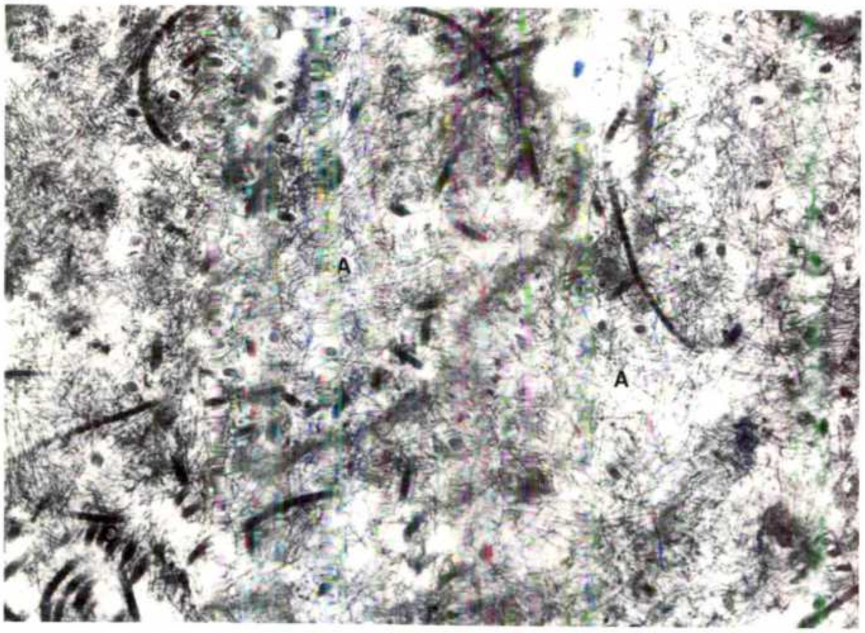

FIGURE 4. Details from the same section as Fig. 3 to demonstrate straight, non-branching amyloid fibrils (A) (× 25,025).

kidney, psoriatic skin and clinically uninvolved skin. Sections when stained with lead citrate and uranyl acetate showed the presence of characteristic amyloid fibrils in sites corresponding to those seen in light microscopy (Figs 3 and 4).

DISCUSSION

Systemic amyloidosis developing as a complication of long-standing psoriasis has been recognized only in recent years. Moise, Asch & Limbs (1965) reported a case of psoriasis and arthropathy with fatal renal amyloidosis. Similar autopsy cases have been reported by Berger (1969) and Brownstein & Helwig (1970a) associating renal amyloidosis with pustular psoriasis, pustular psoriasis with arthritis and psoriasis with arthropathy respectively. In contrast, Ferguson & Downie (1969) described a case of psoriasis and arthropathy with extensive gastro-intestinal amyloid deposits and MacKie & Burton (1974) described a case of pustular psoriasis with renal amyloidosis, amyloid deposits being recognized in life in both.

Our own patient is of interest in that evidence of amyloid deposits have been found in life in kidneys, gastro-intestinal tract, liver, psoriatic skin and also clinically uninvolved skin. Electronmicroscopical, histochemical and immunohistological methods have demonstrated the presence of amyloid in skin and internal organs and have confirmed that it is of secondary type (AA protein). Brownstein & Helwig (1970b) in their extensive review of secondary amyloidosis, incorporating the previous reports of Michelson & Lynch (1934) and Schilder (1909), concluded that cutaneous lesions caused by amyloid do not occur in secondary systemic amyloidosis. In contrast, deposits of secondary amyloid in skin and internal organs in our patient suggests that amyloid deposition in skin does occur in secondary systemic amyloidosis. Recently, cutaneous deposits of secondary systemic amyloidosis associated with diseases other than psoriasis have also been reported by Westermark (1972) and Rubinow & Cohen (1978). Staining of skin sections for amyloid in cases with long-standing psoriasis and arthropathy, as a routine screen for early detection of secondary amyloidosis, is recommended.

There have been major advances in our understanding of amyloid in recent years. Glenner (1980), in his extensive review, suggests the descriptive term β-fibrillosis for amyloid deposits and amyloidosis and describes it as a deposition of twisted β-pleated sheet fibrils formed from various proteins by several pathogenic mechanisms. He has classified amyloidoses on the basis of their major fibril protein and has suggested the term reactive systemic amyloidosis for amyloidosis occurring as a result of acute recurrent or chronic infections, inflammatory or non-immunocyte derived dyscrasias, or neoplastic disorders, with AA protein as the major fibrillar protein, as was demonstrated in our case. He also advanced the concept of 'amyloidogenic' protein precursor modified by proteolysis for the formation of amyloid fibrils. It seems that chronic inflammation in arthropathic psoriasis, as in rheumatoid arthritis, results in persistent elevation of acute phase reactants, including serum AA component (SAA), the putative precursor of AA protein fibrils (Husby & Natvig, 1974). However, the heterogeneity of human SAA proteins (Glenner, 1980) suggests that possibly not all species of SAA are amyloidogenic and consequently only certain individuals with persistently raised SAA will develop amyloidosis.

Prognosis in reactive systemic amyloidosis is better than that of immunocytic amyloidosis and is dependent on the underlying disorder and rate of progression of amyloidosis. There is no universally successful treatment for reactive systemic amyloidosis, although dimethyl sulphoxide (van Rijswijk *et al.*, 1980), colchicine (Ravid, Robson & Kedar, 1977) and cyclophosphamide (Falck, Skrifvars & Wegelius, 1980) have been used in a few patients with some improvement. It is generally accepted that the treatment of underlying disease slows the progression of reactive systemic amyloidosis. Our own case has done reasonably well now for 20 months with methotrexate, nonsteroid anti-inflammatory analgesics and monthly infusions of human plasma protein. His proteinuria has decreased to 2·1 g/l with a rise in his serum albumin to 32 g/l, and his serum urea, creatinine and creatinine clearance have remained normal. When the underlying disease is controlled, a clinical recovery from reactive systemic amyloidosis is possible (Dikman *et al.*, 1977).

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Immune deficiency and multiple viral warts: a possible variant of the Wiskott-Aldrich syndrome

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SUMMARY

A patient with combined humoral and cell-mediated immune deficiency is reported with several features of the Wiskott-Aldrich syndrome but without eczema or platelet dysfunction. Underlying immune deficiency should be considered in patients who present with widespread viral warts.

The Wiskott-Aldrich syndrome is a rare sex-linked recessive condition, characterized by eczema, thrombocytopenia and susceptibility to recurrent infections, presenting in infancy (Wiskott 1937; Aldrich, Steinberg & Campbell 1954). Usually the serum concentration of IgM is low, whereas the serum IgA and IgE may be normal or raised. Isohaemagglutinins and other antibodies to polysaccharide antigens are low in titre. Cell-mediated immunity and T-cell numbers are reduced (Cooper *et al.*, 1968). There is ineffective thrombocytopoiesis; the platelets are small and show diminished response to stimulation with adrenalin (Ochs *et al.*, 1980). The risk of malignancy, in particular lymphoma or leukaemia, is very high. The median survival for patients aged less than 16 years is 6·5 years (Perry *et al.*, 1980), the cause of death being infection, haemorrhage or malignancy.

CASE HISTORY

A 19-year-old man presented to the dermatology department at the University Hospital of Wales with a 7-year history of extensive viral warts (Fig. 1) and a long history of chronic infections. Ten days after birth he was found to be anaemic and was confined to hospital. At 2-3 years of age he had several attacks of viral croup. In later childhood he developed a sequence of infections including meningitis, recurrent pneumonia, whooping cough, mumps, herpes simplex infection and severe attacks of measles and chicken pox. He received live polio vaccine

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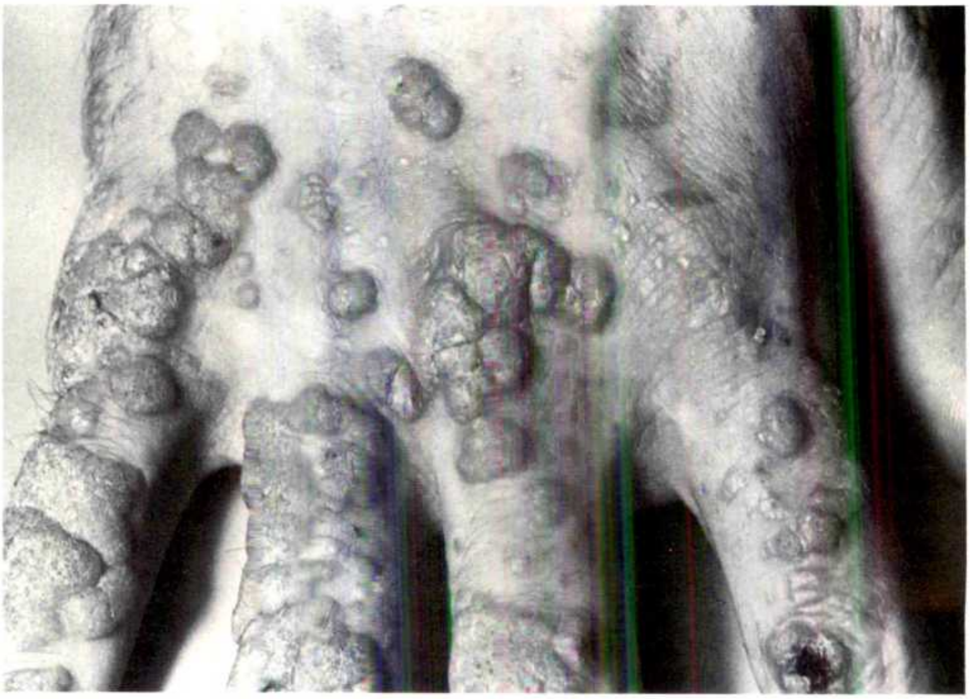

FIGURE 1. Extensive viral warts on the dorsum of the right hand.

without complication; BCG vaccination was not given. On several admissions in early childhood, a leucopenia was noted but repeated bone marrow examinations were normal. Platelets were low at 70 × 10⁹/litre at the age of 16 years, but earlier records were unobtainable. Cystic fibrosis had been excluded by a normal sweat test. He now has severe bronchiectasis with recurrent bronchitis and hæmoptysis. There is no history of bleeding or eczema. His two brothers, sister, parents and mother's male relatives are fit.

On examination there were multiple viral warts over the hands and pityriasis versicolor on the trunk. There were signs of chronic bronchitis, deviation of the trachea and right heart strain. The patient was thin (50 kg) and unwell.

INVESTIGATIONS

Investigations revealed a haemoglobin of 13·8 g/dl, white cell count of 1·7 × 10^9/litre with 69% neutrophils, 18% lymphocytes, 10% monocytes and 1% basophils) and platelets of 60 × 10^9/litre. The urea, electrolytes and liver function tests were normal and the tests for anti-nuclear factor, anti-DNA antibody, smooth muscle and mitochondrial antibodies were negative.

Bone marrow cytology was normal but there was a lymphopenia. Platelet size was normal as was aggregation in response to adenosine diphosphate, collagen, adrenalin and ristocetin. The bleeding time was 6·5 min. Chest X-ray showed displacement of the heart to the left with contraction of the right upper and left lower lobes, with bullae at the left apex. Isotope scan showed a normal liver with some enlargement of the spleen, with an estimated volume of 467 ml.

At biopsy an axillary lymph node was abnormally small (0·3 cm diameter) with very poor development of the lymphoid tissue and an absence of reactive follicles. There was a relative hyperplasia of the intervening sinus histiocytes. The lymphoid cells were almost all small lymphocytes.

Immunological investigation revealed the following findings. There was a positive skin test with candida antigen (1%) but negative responses were obtained with tuberculin 1:1000, *Trichophyton rubrum* (0·5%), mumps skin test antigen and Varidase (5 μg). He did not become sensitized after applications of 2000 μg of dinitrochlorobenzene to the skin for 24 h. With a lymphocyte count of 3×10^8/litre, 26% were T cells and 50% B cells, indicating very low absolute T-cell and low normal B-cell numbers. Because of the low lymphocyte numbers, insufficient numbers of purified cells were obtainable for the conventional stimulation test with phytohaemagglutinin. Therefore a whole blood method was employed, the results being expressed as tritiated thymidine uptake in cells per minute per 15 μl of whole blood (Table 1).

TABLE 1. Lymphocyte transformation in response to phytohaemagglutinin in the patient and a control

	Patient ($0{\cdot}3 \times 10^9$ lymphocytes/ml)	Control (4×10^9 lymphocytes/ml)
Dilution	c.p.m. ± s.d.	c.p.m. ± s.d.
Medium	48 ± 4	30 ± 2
PHA/50	972 ± 222	22,520 ± 311
PHA/250	1242 ± 159	24,427 ± 1019
PHA/1250	197 ± 21	1173 ± 315

c.p.m., Tritiated thymidine uptake in cells per minute.
PHA, Phytohaemagglutinin.

The poor response is due to the low total lymphocyte numbers but the few lymphocytes present apparently respond normally. Anti-lymphocyte antibodies were not detected by complement-dependent cytotoxicity or immunofluorescence tests, nor were platelet antibodies present. Purine metabolism and nucleotide phosphorylase were normal (Giblett *et al.*, 1975). Immunoglobulin concentrations were as follows: IgG 2·83 g/litre (low), IgA 0·55 g/litre (normal), IgM 0·045 g/litre (low), IgE 29 μ/ml (normal). Monoclonal arcs were not seen on immunoelectrophoresis. Antibodies to polysaccharide antigens are given in Table 2. His blood group is B +ve and HLA type is A3, All; B7, w60; Cw3, w7.

TABLE 2. Titres of antibodies to polysaccharide antigens

Antibody	Patient	Normal range
Anti-A	2	8
Anti-Candida	2	8
Anti-*E. Coli*	2	16

MANAGEMENT

The recurrent bacterial chest infections were treated with antibiotics and physiotherapy but are now increasing in frequency and lung function is progressively deteriorating. He has been treated with 50 mg/kg of gammaglobulin daily for 5 days and weekly thereafter and with levamisole 150 mg daily on 1 day of each week, in an attempt to improve his immune status. His pityriasis versicolor infection has cleared with topical econazole. His warts were treated with liquid nitrogen without success. After a year with almost weekly relapses of lower respiratory tract infections there was no sign of clinical improvement. He is now being considered for a trial of treatment with transfer factor.

DISCUSSION

This patient has sufficient features to be considered a variant of the Wiskott-Aldrich syndrome but with atypical features, in particular, the lack of eczema and platelet dysfunction.

The recurrent infections, thrombocytopenia, low levels of IgM and IgG with low levels of isohaemagglutinins and antibodies to the antigens of candida and of *E. Coli* are all consistent with the Wiskott-Aldrich syndrome. In addition T cells were decreased and tests of delayed hypersensitivity were abnormal.

There is no history of haemorrhage or purpura nor any abnormality of platelet function or size. Yet in the classical Wiskott-Aldrich syndrome platelet size, production, survival and response to adrenalin are all reduced, the metabolic activity being lowered with low levels of ADP and ATP (Ochs *et al.* 1980). Asymptomatic carriers show a lesser defect (Shapiro *et al.*, 1978) but this has not been confirmed (Ochs *et al.*, 1980). Our patient's thrombocytopenia may be secondary to hypersplenism and the increased consumption of platelets must be due to factors extrinsic to the platelets themselves. Platelet antibodies were not present in this case and although the spleen was not palpable, isotope scanning showed it be be moderately enlarged. Hypersplenism occurred in one patient studied by Ochs *et al.*, 1980.

In our patient there is no history to suggest an X-linked inheritance. Although there are inconsistencies, this patient best fits the Wiskott-Aldrich syndrome. Formes frustes do occur and spontaneous cases have been reported (Mandl, Watson & Rose, 1968). Evans & Holzel (1970) described a variant of the Wiskott-Aldrich syndrome occurring in a girl with eczema and low IgM. None of those described have exactly the same features as this patient.

Although the underlying defect in the Wiskott-Aldrich syndrome is unknown a fundamental metabolic abnormality has been postulated, which is expressed in platelets by the failure of activating mechanisms (Shapiro *et al.*, 1978). This atypical patient demonstrates that many of the features of the syndrome may occur in the absence of platelet abnormality.

Wiskott-Aldrich syndrome may be corrected by bone marrow transplantation (Parkman *et al.*, 1978). Our patient does not have a compatible sibling donor and was considered unsuitable for transplantation because of the severity of his lung disease. An alternative treatment is with transfer factor, a dialysable extract of leukocytes which has been shown to increase the platelet count and improve the bleeding tendency in the Wiskott-Aldrich syndrome (Mackie *et al.*, 1978). Spitler (1979) treated thirty-two patients with transfer factor and observed clinical benefit with improved survival in 44%. There is anecdotal evidence of benefit from levamisole (Fontan *et al.*, 1976) which has immunostimulant properties but its use in viral warts has been disappointing (Schov & Helin 1977). Thymopoietic drugs such as thymopoietin pentapeptide may prove effective in stimulating cell-mediated immunity.

Warts, a recognized feature of the Wiskott-Aldrich syndrome (Zinn & Belohradsky, 1977), reflect diminished cell-mediated immunity. T-cell deficiency and reduced response to DNCB have been shown to be a frequent accompaniment of viral warts (Chretien, Esswein & Garagusi 1978; Obalek *et al.*, 1980).

This patient, who presented with viral warts, thus has immune deficiency with many features of the Wiskott-Aldrich syndrome but there is no platelet defect. The prognosis of this syndrome is poor and the risk of reticulosis or leukaemia developing is high, perhaps because of decreased immunosurveillance or virus mediated neoplasia (Doll & Kinlen 1970).

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Brief Communication

Calmodulin levels are grossly elevated in the psoriatic lesion

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SUMMARY

Levels of the intracellular calcium receptor, calmodulin, in the psoriatic lesion are more than thirty times higher than normal. By contrast, values in the clinically uninvolved psoriatic skin are unchanged.

Calcium is a major regulator of intracellular metabolism. It is now established that its effects are mediated via the activation of a specific receptor protein, calmodulin. The calcium–calmodulin complex influences enzymes such as adenyl cyclase, phosphodiesterase, protein kinases and phospholipase A_2 (Means and Dedman, 1980; Cheung, 1982; Wightman, Dahlgren & Bonney, 1982) and physiological processes such as endocytosis (Salisbury *et al.*, 1981) and cell division (Chafouleas *et al.*, 1982). Although it is known that calmodulin is present in mouse (Murray & Rogers, 1978) and pig (Iizuka *et al.*, 1982) epidermis, no values have been reported for human skin and possible changes in the dermatoses remain wholly unexplored. Since two prominent features of psoriasis, hyperproliferation and glycogen accumulation, would seem to be calmodulin-dependent, we have now measured calmodulin levels in psoriatic lesions, psoriatic 'uninvolved' skin and in the skin of healthy controls.

Patients were selected who had chronic, stable, plaque psoriasis and who had been untreated for at least 1 month. Controls were paid volunteers approximately matched for sex and age. Biopsies (3 mm diameter, about 0·2 mm thickness) were cut freehand using a razorblade in conjunction with a metal guard; in the case of the patients, one biopsy was taken from a lesion and one from a clinically normal site at least 20 cm from any plaque. All specimens were homogenized in 500 μl of a solution containing 50 mmol/l tris, 3 mmol/l $MgCl_2$ and 1 mmol/l dithioerythritol at pH 7·8, using an all-glass Potter-type homogenizor. The homogenate was centrifuged at 1000 ***g*** for 10 min and aliquots of the supernatant removed for DNA assay according to Kapuścińsky and Skoczylas (1977). The remainder of the supernatant was heated at 65°C for 30 min to destroy endogenous proteases, and calmodulin was determined using the calmodulin [^{125}I]-RIA kit supplied by NEN Chemicals GmbH (Doorn, NL). Levels were expressed as ng calmodulin per μg DNA.

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TABLE 1. Calmodulin levels (ng per μg DNA) in normal and psoriatic skin

	Control	Psoriatic lesion	Psoriatic uninvolved
	23	100	8
	1	335	21
	16	109	8
	12	1221	11
	8	548	19
	6	116	7
	6	200	7
	6	230	22
	12	28	19
		31	8
		691	6
			11
Mean	10·0	331	12·2
±s.d	±6·2	±358	±5·9

The results of the individual measurements are shown in Table 1. It is seen that the mean value for the psoriatic lesions is about thirty times higher than normal; the 'uninvolved' psoriatic skin, however, is not significantly different from the controls (Wilcoxon ranking test, $P > 0{\cdot}1$).

The grossly increased calmodulin content of the lesions, although obviously not the primary genetic expression of psoriasis, may well be of importance in the development and maintenance of the overt lesion. The numerous points at which calmodulin interacts with cellular metabolism makes it unwise to speculate in detail regarding pathogenetic pathways; however, it is tempting to extend the hypothesis of Voorhees and Duell (1971) by postulating either a direct influence on cyclic nucleotide levels or the modulation of protein kinase activity. Clearly more basic data are needed before this area of metabolic control can be clarified.

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Society Proceedings

B.A.D. Investigative Group Meeting, Keele, September, 1982

SUMMARIES OF PAPERS AND POSTERS

Circulating T cells in cutaneous T-cell lymphoma—a study using monoclonal antibodies against normal and malignant T cells

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Circulating mononuclear cells from eight patients with cutaneous T-cell lymphoma (CTCL) were examined using the OKT series of mouse monoclonal antibodies against T-cell differentiation antigens, and two mouse monoclonal antibodies, BE1 and BE2, which selectively react with CTCL cells, EB virus-transformed B cells and some long-term T-cell lines. Of these patients, three had classical Sézary syndrome, two patients had tumour stage skin disease with lymph node involvement and clinical evidence of organ involvement, and three patients had generalized plaque stage disease. In addition, blood from four patients with widespread psoriasis, and from one patient with erythrodermic photosensitive eczema associated with abnormal circulating Sézary cells, was also examined.

Mononuclear cells were separated from freshly collected heparinized blood by velocity sedimentation of Ficoll-Hypaque. The cells were then used in an indirect immunofluorescence technique employing a microtitre plate assay. The OKT antibodies, OKT1, OKT3, OKT4, OKT6, OKT8 and OKT10 were used in a dilution of 1 in 100. BE1 and BE2 were used in a 1 in 500 dilution. A fluorescein-conjugated rabbit anti-mouse anti-serum (Meloy) at a 1 in 40 dilution was used as the second layer.

In all eight patients with CTCL, cells reactive with either BE1 or BE2 were detected. In the three patients with generalized plaque stage disease, cells reactive with BE1 (14–33%) were identified. In one patient 18% of the cells were also reactive with BE2. In these patients the total T-cell count ($OKT1^+$, $OKT3^+$) and the helper/suppressor T-cell ratio were within normal limits.

In the two patients with tumour stage disease and systemic involvement, circulating cells reactive with both BE1 and BE2 (18–50%) were identified. In one patient there was a major expansion of the helper ($OKT4^+$) T cells and in both patients, cells expressing an immature T-cell phenotype (reactive with OKT6 or OKT10) were observed.

In the three patients with Sézary syndrome, large numbers of cells reactive with BE2 (33–59%) were identified and this was associated with an elevation of the total T-cell count and of the helper T-cell population.

In the four patients with widespread psoriasis, no abnormality was found in the total T-cell count or in the helper/suppressor ratio and no cells reactive with BE1 or BE2 were identified. In the patient with the photosensitive eczema Sézary syndrome, a marked elevation of suppressor T cells ($OKT8^+$) was observed, but no cells reactive with BE1 or BE2 were seen.

This study demonstrates the diagnostic value of the antibodies BE1 and BE2 in CTCL as they are able to detect circulating malignant T cells at a stage in CTCL where no other abnormality of the circulating T-cell sub-populations is evident. The study also demonstrates that Sézary cells detectable by light

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microscopy in the blood of patients with benign dermatoses can be differentiated from the Sézary cells of CTCL.

Defective lymphocyte cyclic AMP responsiveness in atopic eczema

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Peripheral blood lymphocyte cyclic adenosine monophosphate (cAMP) responsiveness to the agonists isoprenaline, histamine and prostaglandin E_2 were examined in fourteen patients with atopic eczema and fourteen non-atopic controls. This was performed both in the presence and absence of the phosphodiesterase inhibitor ICI 63197.

After separation of the cells using a Ficoll–Hypaque gradient and dilution to 10^6 cells per ml the lymphocytes were stimulated with isoprenaline (10^{-5}M and 10^{-3}M), histamine (10^{-4}M) and prostaglandin E_2. This was carried out in duplicate at 37°C for 10 min followed by immersion into boiling water for a further 10 min to stop the reaction. Cyclic AMP levels were estimated, in duplicate, by radio-immunoassay.

Basal cAMP levels were similar in both groups. In the presence of the phosphodiesterase inhibitor there were decreased cAMP responses to isoprenaline and prostaglandin E_2 in the atopics with a normal response to histamine. In the absence of ICI 63197 there were very significantly impaired cAMP responses to all agonists, including histamine.

This observation of decreased cAMP responsiveness to all three agonists in the absence of phosphodiesterase inhibitor is in support of increased lymphocyte phosphodiesterase activity in atopic eczema. Similar findings of enhanced mononuclear leukocyte phosphodiesterase activity have been described by Chan *et al.* (1981). Defective responses to beta adrenergic stimulation both in the presence and absence of the phosphodiesterase inhibitor would, in addition, appear to reflect beta receptor dysfunction but this was not found to be selective for the beta-adrenoceptor agonist.

Both mechanisms may be important in the pathogenesis of atopic eczema allowing a new approach in terms of therapy.

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IgG_4 in atopic eczema

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Patients with atopic eczema (AE), many of whom have respiratory allergy, have high total serum IgG_4 levels when compared with patients with atopic asthma and/or rhinitis (A/R) and healthy controls. We have confirmed this by both radio-immunoassay and radial immuno-diffusion. Sequential testing of the IgG_4 levels in AE patients showed the levels to remain relatively constant despite fluctuation of the disease.

We have performed studies of IgG_4 antibodies to five specific allergens (grass pollen, house dust mite (HDM), cat epithelium, egg, cow's milk) using a radio-allergo-sorbent test in thirty-two patients with AE, twenty-eight A/R patients, and a large control group. In general, the specific IgG_4 levels were higher to foods than inhalants (cf. IgE) in all groups (Barnetson *et al.*, 1981). The AE patients had significantly higher IgG_4 levels than the A/R group to HDM, cat epithelium and egg allergens, but not to grass pollen or milk. Both atopic groups had significantly higher IgG_4 levels to HDM, cat epithelium and egg allergens than the controls, and the A/R group had significantly higher levels to cow's milk but not to grass pollen.

The role of IgG_4 in atopic eczema remains to be elucidated, though there is so far no convincing evidence for IgG_4 being a second 'anaphylactic' antibody, or a blocking antibody.

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Immunity and dermatophytosis caused by *T. quinckeanum* in mice

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Experimental dermatophyte infections have been carried out in mice in an attempt to establish the immunological basis of dermatophytosis due to *T. quinckeanum.*

Various strains of mice have been used to illustrate differences in susceptibility to infection. Balb/c mice developed a rapid and pronounced dermatophyte infection on the site of inoculation. Lesions also extended progressively but more slowly in AKR and C3H mice. However, strains CBA and C57BL/6 were relatively resistant to infection. Spleen and lymph node (LN) cells were analysed for *in vitro* mitogenic activity to polyclonal T- and B-cell activators, as well as to specific dermatophyte antigens. In primary infection LN cells showed a significantly reduced response to concanavalin-A, and phytohaemagglutinin, whereas in the spleen only a slight reduction was observed. On the other hand, LPS-induced lymphocyte transformation was unimpaired in LN and spleen cells. The mitogenic response of LN to dermatophyte specific antigens was demonstrated as early as 6 days after infection. In a secondary infection there was only a marginal reduction in the response of LN to T-cell mitogens. This result suggests that suppression of some T-cell activities may be involved in a primary infection. The specific antibody response to fungal antigens was tested by counter-immunoelectrophoresis, plaque-forming cell assay and ELISA. No response was detected for primary infection. However, specific antibody response after a secondary infection was demonstrated.

In addition the role of Langerhans cells and of other non-specific factors in dermatophyte infections was discussed.

Razoxane therapy of psoriasis: comparison of T-cell subpopulations using monoclonal antibodies in treated and untreated patients

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The benefits of razoxane in the treatment of moderate to severe psoriasis are now well established. Apart from its direct effect on epidermal cell proliferation the immunological effects of razoxane should also be considered.

Total T-lymphocyte numbers as measured by E rosette techniques have been found to be reduced in psoriasis. Suppressor T-cell function estimated by concanavalin A inducible suppressor cell function has been reported to be low in some studies though normal in others.

In our study of thirty psoriatic patients half had been established on long-term razoxane therapy and the rest were receiving conventional treatment.

Patients were grouped into three classes, viz., mild, moderate or severe, depending on disease activity and percentage skin involvement.

Total T-cell, helper and suppressor cell populations were identified by monoclonal antibodies UCH T1 and OKT3, OKT4 and OKT8 respectively, and labelled with a secondary fluorescent antibody. Computerized fully automated cell quantification was performed by a fluorescence activated cell sorter.

The results obtained failed to indicate any significant reduction in the total T-cell or suppressor T-cell numbers in patients with psoriasis. Long term razoxane therapy produced no measurable alteration in T-cell subset numbers.

It seems unlikely that abnormalities of T lymphocytes play an important role in the pathogenesis of psoriasis or that razoxane exerts its influence by any primary effect on helper or suppressor subsets.

The characterization of monoclonal antibodies against $\beta 2$ microglobulin and the common determinant of class 1 HLA heavy chains for use in immunoperoxidase labelling of skin tumours

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$\beta 2$ microglobulin ($\beta 2$M), the light chain component of class 1 HLA is present on all normal human nucleated cells. However, recent studies have shown the protein to be undetectable on malignant epidermal neoplasms, using heterologous rabbit anti-$\beta 2$M antisera in an immunoperoxidase technique.

We have characterized the use of three monoclonal antibodies (C21, C23 and B2) against two epitopes of human $\beta 2$M and monoclonal antibody (2A1) against the common determinant of class 1 HLA heavy chains in an immunoperoxidase technique to further investigate the loss of $\beta 2$M from skin neoplasms.

The antisera were employed in dilutions of $1:10$ to $1:10^6$ in an indirect immunoperoxidase technique using an immunopurified rabbit anti-mouse immunoglobulin conjugate. Antisera immunoabsorbed using pure $\beta 2$M were used as controls.

The reactions were performed on cryostat sections from ten pieces of normal skin, fifteen basal cell carcinomas (BCC) and a variety of benign lesions including pilar cysts, seborrhoeic warts and kerato-acanthomas (KA).

Normal epidermis was positively labelled by the monoclonal antibodies C23, B2 and 2A1 to a titre of $1:10^4$, $1:10^5$ and $1:10^5$ respectively. Only C21 positively labelled skin at a titre of $1:10$. Strikingly, at all dilutions hair follicles were unlabelled below the level of entry of the sebaceous glands. The BCCs were unlabelled by the four antisera at all dilutions but often they were surrounded by a positively labelled infiltrate. All the benign epidermal lesions were positively labelled including the pilar cysts and KA.

The study illustrates the use of four monoclonal antisera in an immunoperoxidase technique on skin tumours and confirms a 10^5 fold decrease in $\beta 2$M and directly demonstrates the absence of the related HLA heavy chain from the surface of BCCs.

In spite of the positively labelled pilar cysts, the absence of $\beta 2$M from the deeper portions of hair follicles prompts furthers study of benign follicular tumours often histologically similar to organoid BCCs.

Characterization of the glucocorticoid receptor in human skin

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We have previously characterized the cytosolic glucocorticoid receptor in rat skin. This communication describes the assay and characterization of the glucocorticoid receptor in whole human skin, and separately in dermis and epidermis. Skin samples were obtained from amputated breasts and legs. Epidermis was separated from dermis by stretching and gentle scraping. The purity of each fraction was confirmed histologically. Tissues were pulverized in liquid-nitrogen-cooled mortar and homogenized with a Polytron. Centrifugation of the homogenate at 100,000 ***g*** for 1 h yielded a cytosol fraction which was incubated with 15×10^{-9} M (^{3}H)-triamcinolone acetonide in the presence or absence of a 100-fold molar excess of non-radioactive triamcinoline acetonide to assess non-specific binding. Steroid binding was quantified using dextran-charcoal adsorption and Sephadex G-25 chromatography. In whole skin, binding of the steroid to the receptor was complete after 16 h at 4°C. The binding was of high affinity ($K_d = 1{\cdot}4 \times 10^{-9}$M) and low capacity (65 fmol/mg protein). The fluorinated glucocorticoids (clobetasol propionate, triamcinolone acetonide and betamethasone valerate) competed more effectively for the receptor site than natural glucocorticoids. Non-corticosteroids, with the exception of progesterone, had little or no affinity for the receptor. The binding was labile to heating and to proteolytic enzymes, but not to DNA-ase or RNA-ase. The receptor in the dermis and epidermis was similar to that in whole skin in terms of steroid specificity, temperature lability and affinity. These characteristics of the human skin receptor were similar to those we have previously found in the rat. The distribution of receptor was 92·3% in the dermis and 7·7% in the epidermis. Its concentration for each tissue was also related to wet weight DNA and protein content. We conclude that a glucocorticoid cytosolic receptor protein is present in normal human skin, and most of it is present in the dermis. The epidermal receptor, although much less in amount, appears to have similar physicochemical properties to the one in the dermis.

Both progesterone and oestrogen stimulate preputial gland lipogenesis

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Little is known of the role of ovarian steroids in the control of sebaceous gland function. While progesterone has been shown to stimulate the sebaceous glands, oestrogen either has no effect or is inhibitory. The purpose of this study was to compare the effects of these steroids on sebaceous lipogenesis.

The incorporation of (U-^{14}C) glucose into lipids was used as a measure of lipogenesis in rat preputial glands. Glands were removed from adult female rats, and prior to incubation were either (i) chopped or (ii) disaggregated to form a cell suspension (collagenase digestion, 37°C, 40 min). In some experiments, after incubation a discontinuous density gradient of Percoll was used to separate the cells into five populations, representing different stages of maturity (Alves *et al.*, 1982). Ovariectomy produced a decrease in preputial lipogenesis although in some experiments in which chopped tissue was used the results were not significant. Daily administration of either oestradiol benzoate (OB, 2 μg/rat) or progesterone (P, 400 μg/rat) for 7 days produced no significant change in lipogenic activity of chopped glands. However, P increased the lipogenic activity of disaggregated cells, as did OB at a dose of 10 μg/rat. These increases were seen in all cell populations, and after OB the largest increase was in the mature cells of a low buoyant density. A single dose of OB (10 μg) also increased lipogenic activity of isolated cells when measured 24 h later. Neither steroid affected lipogenic activity when added to the incubation medium.

These results confirm that progesterone is able to stimulate sebaceous lipogenesis and indicate that oestrogen on the preputial glands is related to their specific role in pheromone production, or whether it has similar actions on other sebaceous cells remains to be seen.

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The investigation of hirsutism

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The main concern when dealing with a hirsute patient is to exclude serious pathology or conditions requiring specific treatment. Detailed investigations have been performed in 180 pre-menopausal hirsute females in whom, surprisingly, a definitive diagnosis was made in only 10%. However, these tests suggested that the source of increased androgen production was ovarian in 21% and adrenal in 7%.

Seven different biochemical assays performed on two separate occasions in the menstrual cycle are necessary for full androgen assessment. These fourteen assays become particularly cost-ineffective when choice of treatment is taken into account. In only three cases was treatment selected on the basis of the laboratory results.

It would therefore be valuable to exclude those patients in whom full investigation is unrewarding.

Testosterone and sex hormone binding globulin should be measured in every hirsute female, but where serious pathology is suspected from the history and examination, full investigation becomes mandatory.

In this series not a single patient with normal testosterone ($< 2·5$ nmol/l) had raised androstenedione (A_2) while only one patient with normal testosterone had raised dehydro-epiandrosterone sulphate (DHAS). Of the patients with increased testosterone concentration ($> 3·5$ nmol/l), 54% had elevated A_2 and 21% had raised DHAS levels. 87% of amenorrhoeic patients with raised testosterone levels had elevated A_2.

Thus it would appear that full androgen assessment is unnecessary in those patients with normal testosterone levels and regular menstrual cycles.

Because serum androgen levels have only been generally available in the last 5 years, there has been little opportunity to correlate the hormonal levels with the symptoms and clinical findings. Effective use can be made of these tests on the basis of our study.

Enhanced release of lysosomal enzymes from lithium-stimulated neutrophils in psoriasis

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Several studies have shown that neutrophils are highly activated in psoriasis. We have previously shown that lithium salts cause degranulation of neutrophils *in vitro* (Bloomfield & Young, 1982). In this study, we compared degranulation of neutrophils from thirty patients with chronic, relapsing psoriasis who were not receiving intensive treatment to the neutrophils of thirty healthy individuals without a personal or family history of skin disease and who were not receiving any medication. Lithium was added to whole blood immediately after venepuncture after which the neutrophils were isolated using a Hypaque–Ficoll gradient and enzymes were assayed by fluorometric methods. β-glucuronidase was used as a marker for primary granules and vitamin B_{12} binding protein as a marker for secondary granules. The results clearly showed increased degranulation from primary and secondary granules in psoriatic neutrophils compared to controls ($P < 0·001$). As the basal levels of enzymes in both groups did not differ, the results indicate enhanced release of lysomal enzymes and not merely leakage from overloaded granules. However, measurement of other enzymes associated with neutrophil function, myeloperoxidase and catalase showed a significant increase in the psoriatic group ($P < 0·001$). This study confirms our previous findings (Young & Bloomfield, 1982) that psoriatic neutrophils have a greater capacity to release inflammatory mediators and thereby contribute towards epidermal hyperproliferation. Moreover, this system of studying degranulation of whole blood *in vitro* allows assessment in conditions closely similar to those *in vivo* and we propose that the well-documented reports of lithium salts exacerbating psoriasis can be explained in part by enhanced release of lysosomal enzymes as recently suggested by Heng (1982).

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Comparison of antipyrine metabolism and indocyanine green clearance in psoriatics receiving methotrexate and psoriatics on topical therapy only

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The long-term administration of methotrexate (MTX) to control debilitating psoriasis can cause hepatic fibrosis and cirrhosis. The incidence rises as the total dose of MTX increases. Biochemical tests are unreliable at detecting liver damage and at present liver biopsy is the only reliable method.

The role of indocyanine green clearance and antipyrine (AP) metabolism was studied as a method to detect subclinical hepatic damage. Indocyanine green clearance (IGC) measures hepatic blood flow, and AP metabolism is an index of the liver's oxidative capacity. Twelve psoriatic patients receiving MTX and a similar number who were receiving topical therapy only (as controls) were studied.

The IGC was significantly reduced in the patients receiving MTX and the clearance decreased with increasing MTX dosage. The metabolism of AP was reduced in the MTX-treated group and the reduction correlated with increasing MTX dose.

These two dynamic pharmacological tests of hepatic function may prove to be of value in the follow-up of psoriatics receiving MTX and in identifying those patients who are at risk of developing fibrosis and cirrhosis.

The detection of leukotriene B_4-like material in psoriasis

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Leukotriene B_4 (LTB_4) is a product of arachidonic acid metabolism via the 5-lipoxygenase pathway. It is one of the most potent polymorphonuclear (PMN) chemotactic and chemokinetic substances known. In this investigation we have detected LTB_4-like material in involved skin of patients with chronic plaque psoriasis, using a skin chamber technique.

Skin of normal individuals and of the involved and uninvolved areas of patients with untreated psoriasis was carefully abraded to the glistening layer under magnified vision. Skin chambers were applied to abraded sites and filled with phosphate buffered saline. Fluid was sampled after 5 and/or 35 min, acidified and partitioned with ethyl acetate. Organic phase residues were subjected to an agarose microdroplet chemokinesis assay (Smith & Walker, 1980). Samples from involved skin significantly stimulated the movement of PMNs (73 ± 23·6% increase in the distance moved by PMNs when compared with spontaneous random movement, mean ± s.e., n = 7). In contrast, little activity was found either in samples taken from uninvolved skin of psoriatics (11·8 ± 5·9% increase, n = 11), or from the skin of normal volunteers (21 ± 9·9%, increase, n = 7). Chemokinetic activity in samples from psoriatic plaque was significantly greater than in those from uninvolved skin of psoriatics or skin of normal individuals ($P < 0{\cdot}05$, Student's t test).

Samples from involved skin of four patients were then purified by silicic acid chromatography (Borgeat & Samuelsson, 1979) and reversed phase high performance liquid chromatography (HPLC) using methanol/water/acetic acid (72 : 28 : 0·01, by vol.) as solvent. Effluent HPLC fractions were collected and tested for chemokinetic activity. In all experiments a significant peak of activity was detected which eluted with the same retention time as that of authentic LTB_4. These results indicate that biologically active amounts of LTB_4 were released from psoriatic plaque.

Our findings suggest that LTB_4 may have a role in the pathogenesis of psoriasis, either in the initial recruitment of neutrophils or maintenance of neutrophil immigration into the involved skin.

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The role of prostaglandins in melanosome dispersion

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It is well known that MSH stimulates melanosome dispersion through a membrane-linked receptor and that this action is mediated through cyclic-AMP. Many other factors also stimulate melanosome dispersion, and some of these, e.g. catecholamines, are known to act through cyclic-AMP. We have investigated the effects of prostaglandins on melanosome dispersion and have compared their action with that of MSH.

Effects of several prostaglandins, namely PGE_1, PGE_2, $PGF_1\alpha$ and $PGF_2\alpha$, the prostaglandin precursor arachidonic acid, and also prostacyclines PGI_2 and $6\beta PGI_1$ were examined on the melanophores of *Anolis carolinensis* using the *in vitro* rate method of bioassay (Carter & Shuster, 1978).

The prostacyclins, PGI_2 and $6\beta PGI_1$, had no effect on melanosome dispersion, nor on the MSH dose–response. The prostaglandins $PGF_1\alpha$ and $PGF_2\alpha$ also had no effect, although arachidonic acid produced a weak response with a potency of 10^{-5} to 10^{-6} that of α-MSH. On the other hand, the prostaglandins E_1 and E_2 stimulated melanosome dispersion (potency range 2×10^{-3} to 4×10^{-4} that of α-MSH) with PGE_2 being the more potent. It has been suggested that prostaglandins may mediate the effect of MSH in melanosome dispersion (van de Veerdonk & Brouwer, 1976). However, this seems unlikely in *Anolis* in view of the synergistic action of PGE_2 and α-MSH when given together. Furthermore, indomethacin (0·1–1000 μM), an inhibitor of prostaglandin synthesis, did not affect the action of MSH. While the action of MSH requires the presence of calcium and the melanophore response is directly related to the extracellular calcium concentration, the action of PGE_2 was calcium-independent. The prostaglandins E_1 and E_2 therefore stimulate melanosome dispersion in *Anolis carolinensis* but act differently from MSH. The action of MSH is mediated through cyclic-AMP and is calcium-dependent. The action of prostaglandins however were calcium-independent, but whether their action is mediated through cyclic-AMP has yet to be elucidated.

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Alpha- and beta-adrenoceptor responsiveness in atopic eczema—*in vivo* observations

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Defective beta-adrenoceptor responsiveness and increased alpha-adrenoceptor responsiveness may be of importance in the pathogenesis of atopic eczema (Szentivanyi *et al.*, 1980). Most investigators have empoyed *in vitro* studies, the results of which have been inconsistent. An *in vivo* model for assessment of adrenergic responsiveness in man was therefore designed.

In the skin of healthy subjects, inhibition of mediator-induced inflammatory responses by local administration of both alpha-and beta-adrenoceptor agonists may be demonstrated. There is evidence that beta-adrenoceptor function in skin may parallel beta-adrenoceptor function in the lung in atopic asthmatic subjects.

A double-blind study has been performed in which twenty patients with atopic eczema and twenty non-atopic subjects were given intradermal injections of coded solutions containing histamine (0·75 μg) alone, or mixed with noradrenaline (0·3 μg) or salbutamol (100 μg) at marked sites, in random order, on the forearm. Injection volume was kept constant at 50 μl per site and weal volumes were measured 12 min after injection.

Both salbutamol and noradrenaline caused significant inhibition of the histamine-induced weal response ($P < 0{\cdot}001$) in each group of subjects. However, there was no significant difference between the two groups when their relative alpha- and beta-adrenoceptor responses were compared.

These *in vivo* observations are not consistent with reported *in vitro* observations which have suggested an imbalance in alpha- and beta-adrenoceptor responsiveness in atopic eczema. This model may prove to be a useful tool in the future investigation of the adrenergic mechanism in atopy.

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Raised epidermal phospholipase A_2 activity in the uninvolved skin of psoriasis

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Uninvolved psoriatic epidermis incorporates less (^{14}C) acetate, in proportion to (^{32}P) orthophosphate, into glycerophospholipids than normal epidermis (Summerly *et al.*, 1978). This could be due to defective incorporation of fatty acids into the phospholipids or increased rate of removal of fatty acids from phospholipids. We chose to test the second hypothesis.

Skin was removed from uninvolved areas in patients suffering from psoriasis using ethyl chloride spray anaesthesia. Skin was also removed from corresponding areas of normal individuals. Epidermis, separated from dermis by heating at 50°C for 3 min, was homogenized in a glass tissue grinder. Phospholipase A_2 activity was estimated by measuring the rate of release of labelled fatty acid when the epidermal homogenate was incubated with 2-(^{14}C)olelyl-1-palmitoyl phosphatidyl choline according to the method of Freinkel and Traczyck (1980).

In samples of uninvolved psoriatic epidermis, the mean phospholipase A_2 activity, expressed as nmoles fatty acid released per mg protein per hour, was increased compared with control epidermis ($7{\cdot}9 \pm 1{\cdot}2$ *vs* $2{\cdot}2 \pm 0{\cdot}4$; $P < 0{\cdot}01$).

We believe that this is the first demonstration in uninvolved psoriatic epidermis of increased phospholipase A_2 activity. Enhanced activity of this enzyme would lead to increased concentrations of free arachidonic acid. We think this finding is important, not only because the concentration of arachidonic acid is the rate limiting step in the biosynthesis of prostaglandins and leukotrienes, but also because lysophospholipid (the other product of phospholipase action) is inflammatory and vasoactive (Vadas, 1982).

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Ethyl lactate as a treatment for acne

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Since 1972 there have been a few reports of the efficacy of ethyl lactate in acne, but detailed studies of its mode of action have not been described. We have studied an alcoholic lotion containing ethyl lactate, and have established that this material when topically applied to the skin can accumulate in the sebaceous glands, is hydrolysed to ethanol and lactic acid, and thereby lowers the skin pH. Under such conditions the growth of recoverable *P. acnes*, an anaerobic bacterium that is strongly implicated in acne pathogenesis, is inhibited, and the hydrolysis of sebum to free fatty acids by bacterial lipase is greatly impaired.

The activity of ethyl lactate in controlling follicular events that are involved in acne matches that described by others for benzoyl peroxide. Ethyl lactate, however, possesses no adverse reactions (e.g. sensitization, potential skin irritation or skin scaliness). Clinical efficacy of the ethyl lactate lotion matches that of benzoyl peroxide.

'Open' comedones are closed

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Proponents of the duct obstruction theory of acne claim that open comedones (blackheads) play no part in the pathogenesis of inflammatory acne because they are not blocked functionally.

In order to test the validity of this observation a new modified photometric technique for the measurement of sebum excretion rate has been developed. This has made possible the measurement of sebum excretion from individual pilosebaceous units and permitted comparison with neighbouring pilosebaceous units.

In 200 samplings on scapular skin of fifty patients sebum excretion was measured over a 2 h period, at the end of which a 'follicular pattern' was still evident on the sampling plates. The presence of open comedones was checked photographically, and by both surface and deep biopsy with conventional histology by an observer who was unaware of the sebum excretion result.

Using this technique the sebum excretion rate of open comedones has been shown to be significantly lower ($P < 0{\cdot}01$) than the sebum excretion rate of neighbouring unaffected pilosebaceous units. This suggests that open comedones are functionally closed.

Papular eruption with elimination of papillary oedema: immunopathological studies

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Papular eruption with elimination of papillary oedema (PEEPO) is a recently defined clinicopathological entity in which pruritic urticated papules of endogenous origin exhibit a sequence of histopathological changes characterized by trans-epidermal elimination of papillary oedema (Russell Jones, 1982). Although this process produces morphological changes indistinguishable from eczema, the earliest phase of this inflammatory process is exclusively dermal and consists of a predominantly mononuclear cell infiltrate surrounding upper dermal vessels associated with papillary oedema and variable red cell extravasation. Fifteen patients were studied to establish whether circulating immune complexes (CICs), complement activation and deposition of immuno-reactants are involved in the pathogenesis of this disorder. Two assays were employed for the detection of CICs. Elevated levels were found in sera from ten out of fifteen cases using monoclonal rheumatoid factor binding assay (MrFBA) but in only four out of fifteen case cases using C1Q binding assay (Pussell *et al.*, 1978). Measurement of CH50, C4 and C3 revealed no evidence of hypocomplementaemia in serum samples, and C3 conversion products were detected infrequently in plasma (EDTA) samples (Whicher *et al.*, 1980). IMF of lesional skin revealed no consistent pattern of immunoglobulin deposition: C3 and fibrin were detected in three and six cases respectively. In conclusion, evidence that complement is involved as an inflammatory mediator was not obtained, but the presence of non-complement fixing CICs indicates that humoral mechanisms may be important in this disorder. These findings are analagous to those described in 'lymphocytic' varieties of cutaneous vasculitis (Mackel *et al.*, 1979).

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The application of phase-contrast and dark-field microscopy to the examination of ointments and creams

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The *in situ* examination of inclusions in ointments and creams by standard transmittance light microscopy often proves difficult due, in part, to the low differences in refractivity between inclusions and matrix, unless the latter is water. The use of selective staining procedures or attempted isolation and recovery of such inclusions for examination may yield only limited insight into their actual state in the intact formulation. We have found the techniques of phase-contrast and dark-field microscopy, more commonly used for unstained biological specimens, to aid markedly the direct visualization of inclusions in ointments and creams. The following examples illustrate some of our investigations using these techniques:

A number of corticosteroid-containing ointments have been seen to separate into lagoons of steroid-enriched mobile fluid and relatively inert bases, following mild shearing stresses similar to those expected with normal application of the medicaments. Considerable variations in particle size, uniformity of distribution and drug agglomeration have been noted in similar formulations containing particulate drugs from different manufacturers. The markedly uneven drug distributions in some preparations were sufficient to indicate the probability of erratic dosage as discussed by Orr, Hill and Smith (1980). Seville (1977) considered his observations of irregular, localized, toxicity of some dithranol formulations to be related to drug aggregation within the product and we have recorded marked agglomeration in similar preparations. Antifungal imidazoles (and other antimicrobial agents) in creams from different manufacturers varied considerably in particle size which, for such slightly soluble agents, must influence significantly relative drug availability. Structured inclusions were detected within a variety of ointments and creams which were of a liquid crystalline nature.

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Dietary essential fatty acid supplements and atopic eczema

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The effect of dietary essential fatty acid supplements on symptoms of atopic aczema is controversial. We have studied the therapeutic effect of dietary gamma linolenic acid, and conducted preliminary investigations into a possible mode of action.

Methods. We treated sixty adults and thirty-nine children with atopic eczema in a randomized double-blind crossover trial, using varying doses of oral evening primrose oil and placebo for twelve weeks each. Symptoms were assessed by patient (or parent) and doctor at three weekly intervals on a ten point linear scale. Blood was taken for routine biochemical tests, and plasma lipids. In a later study, T-lymphocyte mitogen responses were investigated before and after treatment in four of the original 'responders' using a micro-assay in inverted Terasaki plates.

Results. Symptom scores were analysed using a modified Mann–Whitney test. As assessed by the patients, itch, scaling and their general impression of their condition were significantly improved by evening primrose oil compared with placebo. As assessed by the doctor, the overall severity of the condition was significantly improved. There were no side effects of note.

Preliminary results from the second study suggest that the therapeutic effect observed may be mediated by an effect on T-lymphocyte function, possibly by changes in prostaglandin metabolism.

Effects of etretinate on epidermal cell production and metabolism in normal skin

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The retinoids have marked effects on keratinization and produce striking improvements in scaling and hyperkeratosis in disorders of epidermal differentiation. Their mode of action is uncertain although a variety of biochemical effects have been described and several mechanisms have been proposed. In order to obtain further information concerning the mode of action of the retinoids on the epidermis, the effects of etretinate on the cytochemical profile and thymidine autoradiographic labelling indices have been studied in normal volunteer subjects. Etretinate (1 mg/kg/d) was given to ten normal subjects over a 4-week period and biopsies taken from the upper arm before and at the end of the treatment period. The biopsies were investigated by quantitative cytochemical techniques for glucose-6-phosphate, succinic and lactic dehydrogenase activities using the densitometer module of the Quantimet 720 image analysis computer. Glucose-6-phosphate dehydrogenase and succinic dehydrogenase activities were significantly increased throughout the epidermis. Portions of the biopsies were also examined histometrically to obtain mean epidermal thickness (MET) and were incubated *in vitro* in the presence of tritiated thymidine to obtain autoradiographic labelling indices (LI). Before etretinate the LI in the group was 4·9 ± 1·2 and the MET 60·23 ± 1·07 μm, and after treatment the LI was 6·8 ± 4·2 and the MET was 60·19 ± 1·94 μm. The results indicate that etretinate may act at several points in epidermal intermediary metabolism rather than at a single differentiative step, but it is unlikely to depend on an effect on epidermal cell production for its therapeutic action.

The increased urinary glycosaminoglycans excretion in psoriasis

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Twenty-four hour urines were collected from twelve male patients with severe psoriasis and from eleven male staff of similar ages. Glycosaminoglycans (GAG) were precipitated with cetyltrimethylammonium bromide and measured colorimetrically as uronic acid. Total uronic acid and creatinine were also measured.

GAG excretion was over 60% higher in the patients ($P < 0{\cdot}01$) and the difference was independent of age or renal function. Total uronic acid was 47% higher in the patients showing that greater catabolism of connective tissue was occurring. Cellulose acetate electrophoresis of the GAG displayed similar patterns of components from patients and controls. There was no novel appearance of the GAG found in skin, hyaluronic acid and dermatan sulphate, to suggest that the additional GAG from the patients was derived from their skin lesions. In four out of five patients GAG excretion remained above normal levels after successful topical treatment with tar or dithranol.

These data suggest that increased catabolism of GAG occurs in connective tissues other than the dermis in psoriasis, representing a degree of systemic involvement.

Prenatal diagnosis of oculocutaneous albinism by electron microscopic observations of fetal skin obtained *in utero*

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The principal types of oculocutaneous albinism are autosomal recessive conditions characterized by absent or reduced melanin synthesis. The diagnosis is established by testing for the presence or absence of tyrosinase *in vitro* and by transmission electron microscopy (TEM) of melanosomes which fail to develop normally.

In the present investigation, prenatal studies were undertaken on a fetus at risk from oculocutaneous albinism. The parents were from the Middle East and several family members were affected. The tyrosinase status had not been determined. At 20 weeks gestation, three samples of fetal scalp were obtained *in utero* under direct vision through a fetoscope using techniques as previously described (Rodeck, Eady & Gosden, 1980).

TEM of several hair-bulb melanocytes showed the presence, in each cell, of numerous stage I and II melanosomes. No stage III or IV melanosomes, indicating melanin synthesis, could be detected. A test for tyrosinase activity by incubating a small skin sample in buffered 0·1% DOPA solution for 4 h at 37°C was negative. In samples from three age-matched normal control fetuses, all hair-bulb melanocytes examined showed numerous stage III and IV melanosomes.

After termination of pregnancy at 22 weeks gestation, the fetal scalp hair and eyebrows were found to be white. Further TEM of scalp and eyebrows gave similar results to the antemortem findings. Histological examination of an eye showed no sign of melanin formation in either the uveal stroma, or the pigment epithelium of the iris, ciliary body or retina. Ocular melanogenesis would normally be advanced in this stage of fetal development.

We conclude that oculocutaneous albinism can be diagnosed *in utero* using similar techniques to those previously described for detecting ichthyosis and epidermolysis bullosa.

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The effects of longterm antibiotics given for acne vulgaris on the bowel flora of patients and their families

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Eleven patients with acne vulgaris volunteered for the study. Six were taking erythromycin; five were taking tetracycline. A faecal specimen was obtained monthly from patients and members of their families.

Aerobic faecal organisms were identified and antibiotic sensitivities were assessed. 'O' sero-typing was performed on the *Escherichia coli* (Cooke, 1974).

Pre-treatment tetracycline resistance was high. Our study shows, however, acquisition of tetracycline resistance and some shared 'O' sero-types of *E. coli* by patients and non-antibiotic taking relatives, thus demonstrating an increase in the pool of antibiotic resistance in the tetracycline-taking families.

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Experimental mouse favus—a new animal model for dermatophyte infections

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A reproducible experimental model for dermatophytosis is essential for the investigation of the recruitment and interaction of immune cells throughout the course of the infection. Previously the guinea-pig has proved to be the most widely used animal model. This study describes the assessment of a new mouse model for experimental dermatophyte infections. First, the infectivity of nine different strains of *Trichophyton mentagrophytes* and *T. quinckeanum* was compared. Using the two most promising isolates, genetic susceptibility of inbred strains of mice was investigated. In subsequent work *T. quinckeanum* (NCPF 309) and Balb/C or Balb/K mice have been used. The immune responses and histopathology of a primary infection or reinfection have been compared. This section concerns the histopathological responses. In the primary infection early invasion of hair follicles is followed by the formation of a dense mycelial mass or scutulum in the stratum corneum maximal at 12–14 days. The inflammatory response consists predominantly of neutrophils and there is little evidence of epidermal proliferation. However, if mice are reinfected after spontaneous clearance of the infection (35 days), the secondary infection is characterized by an early epidermal proliferative response, minimal invasion by fungal hyphae and a dense infiltrate of mononuclear cells as well as neutrophils. The peak of the response occurs in 5–8 days. If mice are reinfected at the peak of the primary infection, there is a dense and rapid accumulation of mononuclear cells and neutrophils, but extensive fungal growth occurs despite the inflammatory cells. This response suggests that both enhancement and suppression of the immune response may occur in a secondary infection—a view supported by subsequent immunological studies.

Altered patterns of actin distribution during normal and abnormal epidermal differentiation

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In many cell types, microfilaments composed of actin interact with other cytoskeletal components and the cell membrane to mediate dynamic shape-changes involved in motility and morphogenetic rearrangement. Control of these processes is exerted by means of the ability of microfilaments and microtubules to undergo reversible disassembly and reassembly in response to changes in the intracellular and extracellular environments. In normal epidermis, regulated migration and changes in cell shape are important aspects of morphogenesis, but the mechanism and means of control of these functions remain unknown.

In this study the distribution of actin within normal human epidermis has been investigated, using electron microscopy, staining the frozen sections with nitrobenzo-oxadiazole (NBD), phallacidin, a reagent specific for filamentous actin, and SDS-PAGE of isolated basal, spinous, granular and horny cells.

The results show that increased amounts of actin are present late in epidermal differentiation, and that in the upper spinous and granular layers only, actin is organized into peripherally located microfilament bundles which interact with tonofilaments. The initial appearance of these peripheral bundles coincides with the onset of keratinocyte flattening, occurring at a constant level below the horny layer in all normal epidermis regardless of the variable thickness of the tissue. Comparison of the histological and biochemical data further suggests that microfilaments depolymerize at the transition between the living and horny layers. This phenomenon is not observed in psoriatic epidermis, in which the distribution of actin within the living layers, and its interaction with tonofilaments, are also abnormal.

These data suggest that changes in the amount, distribution and interactions of actin late in differentiation may be involved in the mechanism of keratinocyte flattening and hence in epidermal morphogenesis.

Abnormalities of keratinocyte culture morphology in epidermolysis bullosa

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In order to investigate abnormalities of epithelial adhesion in epidermolysis bullosa (EB) an *in vitro* system involving keratinocyte culture has been used. Samples of non-blistered, non-scarred skin were taken from one adult and three children with junctional EB, two children with recessive dystrophic EB and one child with generalized EB simplex. Keratinocyte cultures were prepared in the presence of 3T3 feeder cells on collagen-coated dishes using the technique of Rheinwald & Green (1975).

After 14–18 days in culture, all specimens showed a distinctive morphological abnormality which was evident macroscopically. With standard and phase-contrast light microscopy, the centres of established colonies were noted to have detached from the substrate and to have formed blebs or 'blisters', whilst the edges remained attached and growing. This change was evident in all EB cultures and was most marked in the junctional variant. Consistent findings were obtained in repeat samples from two patients.

Immunofluorescence studies (Lane, 1982) on 14-day cultures using antibodies directed against the basement membrane-associated proteins (laminin, fibronectin and bullous pemphigoid antigen) showed negative staining in all three types of EB and in controls. Further studies using antikeratin antiserum and monoclonal antibodies to cytokeratin (LP3K and 3A4) showed no difference in staining between the EB keratinocytes and normal controls.

In conclusion, EB keratinocytes in culture display a unique blistering abnormality regardless of clinical type. Electron microscopic studies are in progress to determine the nature of this separation.

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Analysis of the non-ionic detergent-soluble material of human stratum corneum

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Mechanical stimulation of the skin surface in the presence of non-ionic detergent (Triton X-100) containing buffer leads to the release of large numbers of corneocytes. These are essentially intact as judged by electron microscopy (Barton *et al.*, 1980) and do not appear to differ from those seen in skin sections. In particular the outer membrane is clearly seen, suggesting that the supernatant, to a large extent, is composed of intercellular and cell surface material. Immunofluorescence studies using antisera raised to the non-ionic detergent-soluble (NIDS) protein fraction indicate cross reactivity with stratum corneum and cryostat sections of normal human skin (King, Dykes & Marks, 1982). This reaction was not diminished by absorption of the antisera with 8 M urea–mercaptoethanol keratin fractions. Reactivity has also been demonstrated against strips of stratum corneum isolated by the skin surface biopsy method. As the plane of dehiscence with this method is intercorneal it seems certain that these antigens are in part intercellular. Further studies using isolated strips of stratum corneum indicate that these antigens are trypsin sensitive and are at a higher concentration at greater depths in the stratum corneum. Comparison with the naturally occurring IgG and IgM type circulating auto-antibodies to the stratum corneum, using immunofluorescence and haemagglutination techniques, indicates a separate identity for the NIDS protein antigens.

Preliminary analysis by TLC of the lipids released by this procedure indicate some quantitative differences with regard to those lipids extracted from whole stratum corneum. Work is at present being undertaken to identify and substantiate these differences.

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Limited proteolytic cleavage of human epidermal keratin proteins

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The process of terminal differentiation in mammalian skin is characterized by the production of the insoluble protein, α-keratin, in the cells (corneocytes) of the stratum corneum. Keratin is formed as a precursor, prekeratin, in the lower cell layers (basal, spinous and granular) of the epidermis. Prekeratin extracted from epidermis by citric acid-sodium citrate buffer (pH 2·65), may be partially purified by isoelectric precipitation at pH 4. On SDS-polyacrylamide gel electrophoresis this material is found to consist of a group of polypeptides with molecular weights in the range 46,000–70,000. In contrast, keratin extracted from epidermis after extraction of prekeratin (using 6 M urea/0·05 M Tris-HCL (pH 7·2)/2% mercaptoethanol) is found to consist of a group of polypeptides with molecular weights in the range 44,000–66,000 (Bowden & Cunliffe, 1981).

It has been demonstrated that prekeratin and keratin are rich in the amino acids glycine and serine (Hunter & Skerrow, 1982) and that some residues are phosphorylated. We have investigated the protease digestion behaviour of prekeratin and keratin polypeptides and have found the electrophoretic patterns of peptides to be characteristic. For example, digestion of individual prekeratin polypeptides with *Staphylococcus aureus* V8 protease followed by SDS-gel electrophoresis revealed that cleavage occurred to give distinct patterns. Groups of peptides were observed with molecular weights in the range 30,000–40,000, or in the range 15,000–20,000, or usually both. Experiments in which larger amounts of protease were used suggested that 30,000–40,000 peptides were intermediate stages in the cleavage process. By using our previously published techniques of incorporating (^{14}C)-glycine, (^{14}C)-serine, and (^{32}P)-orthophosphate into the high molecular weight polypeptides of prekeratin (Bladon *et al.*, 1982), we have attempted to detect peptides of high glycine, serine or phosphate contents.

A combination of techniques (e.g. Coomassie blue staining, silver staining, and radio-labelling) were used to identify characteristic parts of the original prekeratin polypeptides. Thus some peptides derived from (^{32}P)-labelled polypeptides contained more radio-label than others. Similar peptide patterns were produced by digestion of keratin. These data add to our knowledge of the structure of 'keratin' polypeptides and should throw light on the processes that take place in the conversion of prekeratin to keratin.

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Improvements in the precision of quantitative assessment of fibrinolytic activity in skin

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Lysis of plasminogen-rich fibrin gels by tissue sections has been used to measure plasminogen activator activity (PAA) in a semi-quantitative manner (Hammar *et al.*, 1980). This was an improvement on previous methods which only measured the amount of lysis at one time point irrespective of the kinetics of the reaction (Dodman *et al.*, 1972). We have further improved the method by eliminating the subjective assessment of the degree of lysis inherent in the technique of Hammar *et al.* (1980). Using the same reagents, plasminogen-free fibrinogen (10 g/l) and plasminogen (2 g/l), mixed in constant proportion and allowed to react on coverslips with thrombin-fibrin gels, were allowed to stabilize in humid chambers for at least 30 min. Three serial cryostat sections of skin were picked up onto each coverslip and incubated in humid chambers at 37°C for 10 time points over 1 h. The reaction was stopped by immersing the coverslips in formalin, and the sections were stained with haematoxylin and eosin and mounted on slides. The area of lysis (A_L) and tissue area (A_T) on each section was measured planimetrically on a Quantimet 720 image analysis computer. The degree of lysis was expressed as log $(A_L/A_T)+2$. The mean of three sections was then plotted *vs* log incubation time (t) for each time point. Hammar *et al.* (1980) suggested that the slope of this line is a measure of PAA, viz.,

$$\log\left(\frac{A_L}{A_T}\right) + 2 = a + b(\log t)$$

where a = the intercept on the y axis and b is the slope (= PAA), derived by regression analysis. The estimated log $(A_L/A_T) + 2$ after 30 min incubation of sections through different parts of one biopsy specimen gave a coefficient of variation of 15%. Table 1 shows the estimates of PAA from calf skin of 4 female volunteers. Results represent the regression analysis determinations serially throughout a 4 mm punch biopsy. Table 2 shows the PAA estimate from normal skin excised during minor surgical operations at two other body sites. Precise measures of PAA such as these are required as baselines prior to attempted therapeutic modulation of fibrinolytic activity.

TABLE 1

Volunteer	Age	PAA*	Correlation coefficient
1	40	1·23	0·77
2	31	2·03	0·92
3	25	1·61	0·89
4	21	2·19	0·94

TABLE 2

Site	PAA*	Correlation coefficient
Hand	2·34	0·90
Trunk	2·24	0·75

*Slope of regression line

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The effects of 'low dose' ionizing irradiation on epidermal structure and function

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Tests which reveal the effects of irradiation at low dose rates to the skin are needed to monitor those exposed to radioactivity. In this investigation the effects on human skin of different sub-erythemal doses of X-ray at different times after irradiation have been studied. Patients receiving therapeutic X-rays for malignant disease who had given informed consent received 'superficial X-rays' (90 kV) to four 5 cm² areas on the volar aspects of the arms. At intervals after irradiation the irradiated sites and one control site were investigated as follows: (a) surface replicas were made for subsequent surface contour analysis; (b) corneocytes were removed by a standardized scrub procedure for mean corneocyte area; (c) biopsy for (i) *in vitro* determination of thymidine autoradiographic labelling indices and for (ii) determination of epidermal thickness. The results (Tables 1 and 2) indicate that although structural parameters did not change significantly for either epidermis or stratum corneum there was a marked dose-related reduction in rate of epidermal cell production as judged by thymidine autoradiographic labelling indices. Ideally a non-invasive technique would detect change in rate of epidermopoiesis, and other studies of stratum corneum structure and replacement time are in progress.

TABLE 1. Mean corneocyte area (MCA) after irradiation (μm^2)

Days post irradiation		Dose (Rads)							
		0	10	50	100	200	250	350	450
6	MCA	1113	1160	1213	1226	1050	1091	1035	950
	± SD	± 113	± 88	± 95	± 96	± 166	± 70	± 97	± 70
	n	7	3	3	3	7	4	4	4
12	MCA	1104	1169	1122	1193	1102	996	1037	1020
	± SD	± 157	± 73	± 40	± 61	± 227	± 162	± 165	± 136
	n	6	3	3	3	5	2	2	2

TABLE 2. Effect of different doses of X-ray upon labelling indices and mean epidermal thickness (MET) after 6 days

	Dose (Rads)								
	0	10	50	100	150	200	250	350	450
Labelling* index ± SD	4·95 ± 1·01	3·94 ± 1·97	2·72 ± 0·93	1·43 ± 0·65	0·30 —	1·04 ± 0·72	0·96 ± 0·42	1·125 ± 0·61	1·10 ± 0·78
MET (μm)	34·52	33·19	33·37	34·73	29·30	31·76	31·74	35·52	39·97
± SD	± 4·79	± 9·59	± 4·74	± 5·86	± 5·08	± 3·73	± 1·18	± 4·42	± 5·34
n	9	4	4	5	1	9	5	4	4

*Number of cells labelled by [^{3}H]thymidine autoradiography expressed as a percentage of the number of basal cells.

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Comparative studies of sebaceous gland ultrastructure

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Cattle sebum is apparently formed by lipogenesis and secretion from living cells as well as from degenerating cells, the end-product of a holocrine process (Smith & Jenkinson, 1975). Scrutiny of the mode of sebum synthesis and secretion has been hindered by the lack of an accurate method of determining the rate of secretion, as opposed to excretion, from the gland. A potential chemical marker for study of bovine sebum triglyceride synthesis and output has emerged from the discovery that these triglycerides contain significantly more linoleic acid than those of other bovine tissues; they are unique to the sebaceous gland (Jenkinson, McMaster & Noble, 1982). As a prelude to location of the site of triglyceride synthesis, glandular ultrastructure (which in farm animals is unknown) was determined.

Skin specimens from four cattle, sheep, goats and equines were processed for electron microscopy. Sebaceous gland in blocks from every animal were sequentially sectioned at 1 μm in different directions and ultrathin sections were cut at various levels. A 3-dimensional reconstruction of one gland from a steer and one from a horse was made from a complete series of 5 μm sections, using computer-linked planimetry.

The cells lining the neck and upper portion of the gland were found to be keratinizing. Differentiating cells which were located in the lower part of the lobe gave rise to a column of cell populations at different stages of development. The differentiating cells, which are interconnected by desmosomes, contain membrane-bound lipid droplets closely associated with smooth endoplasmic reticulum and mitochondria.

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Analysis of fatty acid methyl esters derived from isolated human sebaceous glands

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Neutral lipids were extracted from isolated human sebaceous glands and separated by thin layer chromatography. Triglycerides and wax monoesters were eluted from the gel and hydrolysed. Fatty acids were extracted from the hydrolysate and their methyl esters were examined by gas chromatography–mass spectrometry.

The results were in good agreement with surface lipid studies. No esters were found to correspond to fatty acids of chain length shorter than 12:0, and the population lay almost entirely between 12:0 and 18:0. Chain lengths greater than 18:0 were tentatively identified. Many small peaks were interpreted as being the methyl esters of branched-chain fatty acids, and these were found to be associated with both triglycerides and wax monoesters. Larger peaks were found to correspond to unsaturated fatty acids, but these were not analysed further.

These findings support the hypothesis that branched-chain fatty acids are synthesized within human sebaceous cells, and imply that a common cytoplasmic fatty acid pool exists when triglycerides and wax esters are synthesized. Differences between the fatty acid populations of these two classes of esters (as noted in surface lipid studies) may be related to membrane-associated lipid synthesis. The absence of short- and medium-chain fatty acid methyl esters may reflect technical limitations, but it is also possible the such fatty acids are not synthesized in sebaceous glands, since most mammalian fatty acids synthetases will not normally produce them.

Studies of the human stratum corneum

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In animals, sebaceous lipid is present between the squames of the outer stratum corneum (Jenkinson & Lloyd, 1979; Lloyd & Garthwaite, 1982). In some species this intercellular reservoir is the most likely source of the surface lipid, although it is not known if mammalian sebum reaches the surface solely via this route. Topical solvent application removes a significant portion of the bovine corneum including the intercellular lipid and reduces resistance to infection (Lloyd & Jenkinson, 1980). In man, there is little evidence on the actions of soaps, detergents and solvents upon *in vivo* corneum integrity and thus skin surface appearance and corneum thickness were determined prior to investigation of the effects of bathing on the healthy and abnormal epidermis.

Duplicate samples were taken by biopsy after local injection of 2% lignocaine, from areas of skin on the legs of four males and arms of four females, which had not been washed for 7 days or pre-treated. All samples were quenched in liquid nitrogen and either freeze-dried, coated and examined by scanning electron microscopy, or cut at 10 μm at −40° C and stained with methylene blue or Sudan IV for lipid. The remainder of each of the cryosectioned blocks was fixed in 10% formol saline, routinely processed to paraffin, sectioned at 10 μm and stained with H & E. Fifty measurements were made of corneum and living epidermal thickness, and of the number of cell layers present (including sudanophilic layers). The skin surface had a similar appearance to that of the dog. The corneum, which was 22·5 μm thick in the females and 29·4 μm in the males was composed of twenty-two and twenty-five cell layers, respectively. Lipid was found between the outer twelve of these layers in the females and the outer nine in the males. Paraffin processing removed fourteen layers in the females and sixteen in the males and grossly distended the spaces between the remainder. It had no appreciable influence on the dimensions of the living epidermis. The actions of a soap and water bathing routine on the healthy corneum are now being examined.

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Quantification of absorption through damaged skin

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We have previously reported (BAD Investigative Group, 1981) the changes in the water permeability of rat skin after damage and during barrier regeneration. The bi-phasic return to normality in rat skin is more rapid, but otherwise similar to that observed for human skin. Using this information, together with a parallel study of the histology of the repair process we have devised an *in vivo* model for studying the factors governing absorption through physically altered skin.

The model design selected was rat skin altered by fifteen applications of adhesive tape and allowed to recover for 0 and 24 h (during the first phase of water barrier regeneration) and 96 h (second phase) before the application of test material.

The pattern of excretion of tritium was characterized by urine analysis after i.v. dosing of [^{3}H]mannitol. Tritium was rapidly excreted and therefore urine analysis was suitable for assessing rates of absorption through skin. In skin absorption measurements, an aqueous solution of [^{3}H]mannitol was applied under occlusion to the skin and hourly urine samples collected for 24 h. Adequate urine production was ensured by orally dosing the rats with 10% glucose in normal saline.

[^{3}H]mannitol was slowly absorbed through normal skin (0·42% of topical dose) and damage caused a large increase in absorption rate (52% of topical dose when applied to 0 h recovered skin, 140 times normal). In contrast to the epidermal barrier to water loss, no regeneration of a barrier to [^{3}H]mannitol had occurred by 24 h after damage but by 96 hours a reduction in permeability was apparent.

The model has been used to quantify the absorption through although skin of a range of molecules. Generally the more slowly a molecule penetrates normal skin the greater is the factor of increase through abnormal skin.

The rat skin model will permit assessment of the hazard of chemicals contacting damaged skin and could be used to quantify the maximum possible absorption of drugs through diseased skin.

Morphometric and adhesion studies on the normal human dermo-epidermal junction

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A preliminary study of ultrastructural components of the dermo-epidermal junction in skin from normal men showed a regional variation in anchoring fibril (AF) numbers (Tidman *et al.*, 1982). The investigation has been extended to examine possible sex differences and to see whether measurable adhesiveness of the dermo-epidermal junction could be correlated with structural characteristics, as AF may traverse the basal lamina (BL) and cross the lamina lucida (LL) (Susi *et al.*, 1967).

Skin samples from defined areas on the lower leg, thigh and upper arm from five healthy women (age range 23 to 55 years) were processed for transmission electron microscopy. The population densities of hemidesmosomes (HD), plasmalemmal vesicles (PV) and AF were calculated, and thickness of BL and LL, associated and between HD, were measured as previously described (Tidman *et al.*, 1982). As an indicator of dermo-epidermal adhesion within the LL, the suction blister time (SBT) was measured (Kiistala, 1972) for each of the three pre-defined body regions in five men and eight women.

No sex differences were found for HD, PV and AF counts. Analysis of the pooled data for both males and females showed no significant differences for HD and PV counts or in thickness measurements between body regions. However, there were fewer AF in the arm (34·1 ± 18·3 SD) compared with the thigh (69·7 ± 26·2) or lower leg (73·[illegible] ± 22·2) (P < 0·01). The mean SBT in min was 82·9 ± 27·4 SD for the arm, 91·5 ± 27·4 (thigh), 145·1 ± 38·8 (lower leg). There was no significant correlation between AF counts and SBT in sets of data available from five subjects.

In conclusion the regional difference in AF counts is evident in both males and females. Although the regional variation of AF numbers tended to parallel differences in SBT, our data do not support the hypothesis that AF have a role in LL adhesion.

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Mechanical properties of the dermis in psoriasis

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Psoriasis is usually conceptualized as an 'epidermal disease'. However, the abnormal vascularity and the inflammation ensure that the dermo-connective tissue is also involved. It has been suggested that the rate of collagen synthesis is increased (Dykes, Jenner & Marks, 1982) alongside the epidermal hyperplasia. In this investigation the mechanical properties of the dermis and skin thickness have been measured in the non-involved skin of psoriatic patients and compared to a matched control group of normal volunteer subjects, in order to determine whether any inherent structural differences existed. To measure dermal physical properties, an extensometer of the type described by Gunner, Hutton & Burlin (1979) was used. Under basal conditions patients and controls were tested on identical sites in the same orientation of the extensor aspect of the forearms. Skin thickness was measured by the pulsed ultrasound method (Tan *et al.*,

1982) on similar sites. Measurements were made of the peak forces in three consecutive extensions of 30% of the initial separation of the extensometer tabs. The force after 5 seconds relaxation was also recorded. The uninvolved skin of psoriatics was found to be considerably 'stiffer' than controls (Table 1) although skin thickness did not differ between the two groups (Table 2). The striking difference in mechanical properties, without change in thickness between psoriatics and controls, almost certainly indicates a previously undetected structural alteration of some kind, despite the apparent normality of psoriatic dermal connective tissue on routine microscopy.

TABLE 1. Mechanical properties of skin in psoriatics and controls

		Uninvolved Skin of Psoriatic Subjects	Matched Normal Control Subjects
Mean peak force at 30% extension		450·1 ± 148·2*	308·8 ± 107·4
Mean percentage difference in force after 5 seconds relaxation	Peak 1	32·8	31·9
	Peak 2	25·8	24·5
	Peak 3	25·0	25·8

*p = 0·05

TABLE 2. Skin in thickness

	Uninvolved Skin of Psoriatic Subjects	Matched Normal Control Subjects
Mean skin thickness	0·62 ± 0·09	0·65 ± 0·15

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Increased macrophage activity in psoriasis

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A number of reports have been published on the functional changes in bone-marrow-derived cells in psoriatic patients. These include increases in chemotaxis and phagocytosis by PMN cells and monocytes. It has been postulated that this increase in cell function is an inherent disorder, probably mediated via a decrease in the cAMP/cGMP ratio.

Macrophages present in tissues have characteristics which differ from circulating monocytes (Elias & Epstein, 1968) and these changes in function and biochemical characteristics are associated with a maturation or differentiation process. Current evidence that macrophages are altered in psoriatic subjects is indirect. We have attempted to study macrophages derived from circulating psoriatic monocytes to see if the hyperactivity of psoriatic neutrophils and blood monocytes also occurs in these cells.

Macrophages were obtained by culturing circulating blood cells for 48 h and then washed to remove PMN cells. The mononuclear cells were allowed to grow for a further 4 days when they transformed into macrophages.

The results showed that psoriatic macrophages release more β-glucuronidase and lysozyme than normal cells. Total protein content was also increased in the psoriatic cultures, and so is the number of giant cells formed.

Our results demonstrate that macrophages derived from psoriatic monocytes are hyperactive. The relationship of activated neutrophils, monocytes, macrophages and possibly Langerhans cells to the disease process is discussed.

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Lectin binding studies on normal and abnormal human skin

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Lectins are a group of proteins which bind to specific carbohydrate groups such as those of cell surface glycoproteins, glycolipids and extracellular matrix components. In this study lectins were used to investigate complex carbohydrate in normal skin and in a variety of skin diseases.

Air-dried frozen sections of normal and abnormal human skin were reacted with a range of rhodamine conjugated lectins selected to visualize various carbohydrate configurations. Binding specificity was confirmed by competitive saccharide inhibition.

A characteristic pattern of distribution and intensity was observed for each lectin, viz., wheat germ agglutinin stained the surface and adnexal epithelium, a broad basement membrane zone and the dermis. Concanavalin A and *Lens culinaris* agglutinin also reacted overall but most intensely with the dermis. Soybean and *Ulex europaeus* agglutinins particularly stained the spinous and granular layers and dermal capillaries. Peanut agglutinin, in addition to the spinous and granular layers, demonstrated a narrow basement membrane zone and sebaceous glands. *Bandeirea simplicifolia* lectin I reacted most intensely with the granular layer. *Bandeirea simplicifolia* lectin II gave an intracellular staining of the mid epidermal region. The horny layer did not react with any of the lectins. These data demonstrate that lectin binding sites comprise a series of markers for normal keratinocyte differentiation and can define normal carbohydrate composition in the basement membrane zone and various dermal structures.

The following disorders involving abnormal epidermal differentiation were examined: psoriasis, basal cell papilloma, basal cell carcinoma and squamous cell carcinoma. In general the characteristic lectin binding patterns for each condition reflected the stage of differentiation as judged by other criteria. In basal and squamous cell carcinoma basement membrane zone and stromal alterations were also apparent. These results suggest that lectins are powerful tools in studies on the role of cell surface and stromal interactions in normal differentiation and in pathogenesis.

Response to short term application of dithranol in psoriasis

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We set out (1) to use objective methods to confirm recent reports that psoriasis responds to short term application of dithranol, and (2) to optimise the method for time and concentration. Seventeen male and nineteen female psoriatics aged 14–67 were studied. In all, conventional 0·05–1% dithranol treatment (Ingram regimen) was used for comparison. Response was measured as lesion thickness with Harpenden calipers, and as time to clearing by appearance and feel. Severity of burning was graded 0–3 and its frequency recorded daily. In a confirmatory study thirteen patients applied 2% dithranol for 60 min to one arm. Optimum time of application was found to be 30 min and thirty-six patients had applications of 4, 8, 12 and 16 per cent dithranol for 30 min to uninvolved skin. Burning was unacceptable at greater than 4% concentrations, therefore ten patients applied 4% dithranol to one-half of the body for 30 min. Thirteen patients used 2% dithranol similarly, but only increasing after 3-day intervals to 4% and 8% if there was no burning.

Overall time to reach one-half the original lesion thickness ($t_{1/2}$) and time for them to clear (T) correlated well ($r = 0·88$, $P < 0·001$). Short term treatment failed in two cases because of burning and in three who did not respond. In the remainder both the clinical and objective response were similar. Burning was greater with all three short term treatments ($P = 0·05$, $P < 0·05$, $P < 0·01$; Wilcoxon signed rank).

We conclude that short treatment with dithranol was as effective as conventional treatment in most cases. The optimal treatment was 30 min with 2–4%, and burning is the main defect.

TABLE 1. Conventional vs short treatment with dithranol

	2%, 60 min	Conventional	4%, 30 min	Conventional	2–4–8%,	Conventional
t 1/2 (days)	5·8 ± 2·6	5·4 ± 2·6	6·6 ± 3·8	6·9 ± 2·5	5·9 ± 2·8	8·5 ± 2·6
T (days)	11·0 ± 3·9	11·1 ± 3·9	13·4 ± 4·0	13·2 ± 2·9	15·8 ± 4·5	14·9 ± 4·3
Number of patients	13		10		13	

The treatment of rosacea with 13-*cis*-retinoic acid

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Nine patients (at present) have received 13-*cis*-retinoic acid orally in a dose of 0·5 mg/kilo/day for 6 weeks. The patients comprised four females and five males with an age range of 27–59 years and were selected on a basis of severe long-standing rosacea which had not responded to oral tetracycline. Sebum excretion rates (SER) were performed prior to starting therapy and at 6 and 10 weeks. Assessment was based on lesion count, linear analogue scale and sequential photography. Serum lipids were assessed prior to therapy and at 6 and 10 weeks. Full biochemical, haematological and ophthalmic checks were performed prior to and during the study. All topical and systemic therapy was discontinued 4 weeks before entry into this study.

In all patients there has been a significant improvement judged by falling lesion counts and visual analogue scale measurements. SER levels were all within the normal range prior to therapy and in every case fell to zero at 6 weeks. By 10 weeks the SER had returned to 60% of pre-treatment levels.

All patients experienced side-effects. These were cheilitis, epistaxis, conjunctival irritation (2), dermatitis (1) and raised triglycerides (2).

We conclude that 13-*cis*-retinoic acid may be of value in the management of severe resistant rosacea provided patients are willing to tolerate side-effects.

Effects of an arotenoid (Ro13-6298) in the treatment of multiple solar keratoses and squamous cell epitheliomata

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Retinoids have an anti-neoplastic effect for some experimentally-induced tumours although the mode of action is uncertain. In man, etretinate has been found effective in the treatment of solar keratoses (Moriarty *et al.*, 1982) and a potent new retinoid (Arotenoid Ro13-6298) has also been shown to have an anti-neoplastic action on cutaneous malignancies (Tsambos & Orfanos, 1982). In this study patients with solar keratoses and/or squamous cell epitheliomata have been treated with Ro13-6298 for 28 days with an oral dose of 1 μg/kg/day. The lesions of fifteen patients (mean age 67) were carefully recorded on a body chart and by photography before and after treatment. Biopsies of lesions and of uninvolved skin of the inner aspect of the upper arm were taken before and after treatment. These were examined by histometric techniques using the Quantimet 720 image analysis system for epidermal thickness and by tritiated thymidine autoradiography for labelling indices. In ten patients there was a striking clinical improvement with a reduction in the number, area and thickness of lesions. In five patients there was little change. It was noticed fortuitiously that in some patients seborrhoeic warts also responded well to the treatment. The side-effects experienced were similar to those encountered with other retinoids. Dryness of the lips, pruritis and exfoliation of the palms and soles were noticed in ten subjects and in three of these a distinctive eczematous rash occurred. Uninvolved epidermis showed a significant increase in epidermal thickness (see Table 1) as noted previously in patients with disorders of keratinization treated with etretinate (Marks, Finlay & Holt, 1981). There was also a small increase in the thymidine autoradiographic labelling indices in the uninvolved epidermis of Ro13-6298 treated patients (Table 1). The Arotenoid Ro13-6298 is a potent retinoid with marked effects on epidermal differentiation and produces resolution in a substantial proportion of preneoplastic and neoplastic lesions of the epidermis.

TABLE 1. Epidermal thickness after Ro13-6298 treatment

	Mean epidermal thickness (μm)	Labelling indices (per cent)
Pre-treatment	57·22 ± 13·87	5·55 ± 3·02
Post-treatment	75·69 ± 19·17	7·22 ± 4·92
Significance	0·05 > p > 0·02	N.S.

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The effect of testosterone on maximal sweat rate in eunuchs

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The maximum rate of cholinergic induced sweating is greater in men than women and this difference occurs at puberty (Rees & Shuster, 1981). The role of androgen was therefore studied by measuring maximal sweat rate in eleven eunuchoidal men with prepubertal hypogonadism. At the time of measurement six patients were untreated and five were receiving androgen replacement. Four of the six untreated patients were then treated with androgen and sweat rates were obtained in the subsequent months. Sweating was induced by iontophoresis of 0·2% pilocarpine and was collected over a 15 minute period onto 3 cm filter paper discs inside aluminium capsules stuck to the skin, as described elsewhere (Johnson & Shuster, 1969). Sweat rates in the untreated patients were similar to those previously found in prepubertal boys and girls (corrected for surface area) and normal women. By contrast, with one exception, sweat rates were higher in the treated group. There was a good correlation between the post-treatment sweat output and post-treatment plasma testosterone concentrations ($P < 0{\cdot}01$). There was also a good correlation ($P < 0{\cdot}01$) between the sweat response to testosterone and the pre-treatment state as measured by the pre-treatment plasma testosterone. Of the four patients studied before and after treatment, three showed an increase in sweat rate and the one in whom the sweat rate did not increase had only a small increase in plasma testosterone.

These findings show that the response is related both to the severity of the condition before treatment and to the magnitude of the rise in circulating testosterone after treatment. There was no correlation between the post-treatment sweat rate and duration of treatment, and the response to androgen appeared to have occurred within the first few months of treatment. The present study indicates that the sweat rate in pre-pubertal eunuchs is similar to that of the adult female and that it increases to the normal male range after androgen. However, it does not allow us to distinguish between a modulating action of androgen and the irreversible gene expression of sweat gland function which appears to occur in normal puberty.

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Lysyl oxidase activity in granuloma annulare and necrobiosis lipoidica

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Granuloma annulare (GA) and Necrobiosis lipoidica (NL) are connective tissue diseases of unknown aetiology. Typically, they present as a dermal lesion consisting of a central area of necrosis with abnormal collagen fibres and a deposition of a PAS-positive, diastase-resistant material. This is surrounded by a region of palisading histiocytes and fibroblasts. Both GA and NL have been linked with diabetes.

Lysyl oxidase oxidately deaminates lysine and hydroxylysine residues of collagen chains, which spontaneously undergo either an aldol condensation or Schiff base cross-linking reactions. In this way the intra- and inter-chain cross-linking between collagen fibres occurs. Cross-links are essential for the stability of collagen chains.

Lysyl oxidase activity was determined by a modification of the tritium release assay from [6-^{3}H]-lysine labelled chick calvaria (Bachra, Melet & Vianden, 1977). The tritiated water released during the assay was extracted with small Dowex 50W-X8 ion-exchange columns, eluted with a small amount of 5%

trichloroacetic acid and counted in a scintillation counter. Early data shows an alteration of lysyl oxidase activity in the fibroblasts cultured from patients with GA and NL when compared with controls. Data from several patients with GA and NL will be given. Also, the consequences of these results in relation to possible treatment will be discussed.

Several enzymes involved in the post-translational modification of collagen, viz., prolyl, lysyl hydroxylase and glycosyl-galactosyl transferase have been assayed. The significance of our findings will be discussed.

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Hydrocortisone 17-butyrate blood levels after percutaneous absorption

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Using a specific radioimmunoassay for hydrocortisone 17-butyrate (HCB) with a threshold of 40 pmol/l (Lee, 1979), we have examined the blood levels of HCB achieved following twice-daily application of 30 g 0·1% HCB ointment (Locoid®) for 4 to 8 days to thirteen in-patients with eczema and 30 g 0·1% HCB cream (Locoid®) for one week to twelve volunteers. Polythene suits occluding 80% of body surface area were worn by six volunteers for twelve hours daily.

Highest HCB levels were found in occluded volunteers (mean ± standard deviation = 3·35 ± 1·89 nmol/l), intermediate levels were found in patients (mean ± standard deviation = 2·13 ± 1·28 nmol/l) and lowest levels were found in unoccluded volunteers (mean ± standard deviation = 1·63 ± 1·37 nmol/l). Blood HCB levels fluctuated rapidly and varied by up to 100% in 2 hours in two patients studied over a 12-hour period. Strenuous exercise for 15 min more than doubled HCB levels in two of three subjects tested.

There was no significant difference in the cortisol levels of patients and occluded volunteers and the mean reduction of cortisol seen in both groups, from 300 ± 79 to 197 ± 93 and from 288 ± 87 to 219 ± 77 nmol/l, respectively, was highly significant ($P < 0{\cdot}001$). Statistically significant reduction of cortisol could be shown in one of six unoccluded volunteers, in three of six occluded volunteers and in five of nine patients. Two occluded volunteers and six patients had subnormal cortisol levels on at least one morning but only one patient remained suppressed throughout treatment. A statistically significant inverse correlation between HCB and cortisol was seen in three patients and two occluded volunteers only. There was a tendency for serum cortisol to rise and blood HCB to fall as patients inproved with treatment.

If the conclusions from this study are applicable to other corticosteroids, the volunteer using polythene occlusion for 12 h daily is a useful model for the prediction of pituitary-adrenal suppression in patients receiving topical corticosteroids.

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Correspondence

Trichothiodystrophy—BIDS, IBIDS AND PIBIDS ?

SIR, The occurrence of ichthyosis in the trichothiodystrophy or BIDS syndrome recently led Dr J.L.Jorizzo and colleagues (*British Journal of Dermatology* 1982, **106,** 705) to propose a possible subset in the form of the IBIDS syndrome.

We have observed a patient with the IBIDS syndrome who also had photosensitivity. A 10-year-old girl presented with short stature and the typical facial features, with micrognathia, prominent ears, bilateral congenital cataracts, impaired intelligence, extensive dental caries, ichthyosis, brittle hair and nails. The hair showed trichoschisis, with alternate banding on polarizing microscopy and cystine deficiency. In addition, 5-min sun exposure was sufficient to induce erythema, oedema and bullae on the exposed areas.

Photosensitivity is not a recognized feature of the BIDS syndrome, but it has recently been reported in a patient with trichothiodystrophy who apparently lacked ichthyosis (Van Neste, 1982).

Do these two cases with photosensitivity allow us to extend the acronym to PIBIDS?

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VAN NESTE, D.J. (1982) Trichoschisis: Light sensitivity and growth retardation. *XVI Congressus Internationalis Dermatologiae, Tokyo 1982.* Case presentations, p. 79.

Hyperkeratotic dermatitis of the palms

SIR, The interesting paper by Hersle & Mobacken (*British Journal of Dermatology* 1982, **107,** 195) on hyperkeratotic dermatitis of the palms surely includes many examples of lichen simplex, alias neurodermatitis. The authors rejected this diagnosis because, *inter alia*, 'almost all patients denied that the lesions itched.' But many patients suffering from lichen simplex do deny itching, though if asked 'how do you rub your skin?' they will often oblige by a demonstration that may include rubbing with the finger nails (the ends or the flat), rubbing on furniture, or using a tool, such as a brush. Even if there has been a complete denial, it may still be possible to detect polishing or bevelling of the finger nails which have been used for this purpose. Why a denial should be necessary is not quite clear. It may be that the vivid sensation is regarded as different from an itch, but more likely, I suspect, is that freedom to rub the skin produces pleasure of such intensity that the patient is loth to be deprived of it.

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Dilution of betamethasone ointment

SIR, Recently Ryatt *et al.* (*British Journal of Dermatology* 1982, **107**, 71) have reported on the bio-availability of betamethasone-17-valerate (Betnovate®) ointment when diluted in emulsifying ointment and have related decreases in vasoconstrictor efficiency to its interconversion to betamethasone-21-valerate. Our investigations into the stability of Betnovate® ointment and various dilutions of it using similar H.P.L.C. techniques and microscopy have revealed data which supplement their findings.

It appears that appreciable changes have been made to the formulation of Betnovate® ointment without public announcement and that material obtained from the manufacturers after 1981 is significantly different in structure and physical behaviour from previously supplied ointment. In the 'old' formulation the betamethasone-17-valerate was dissolved in a mobile liquid, tentatively identified as propylene glycol, and dispersed as droplets in a hydrocarbon base. Aliquots of ointment removed as gently as possible from tubes were found to have an acceptable uniformity of drug distribution. However, due to sampling disturbance and analytical method a coefficient of variation of 4·14% and 3·64% was obtained on two sets of ten 25 mg samples. Orr, Hill & Smith (1980) have pointed to the need to analyse samples in small quantities such that the scale of scrutiny is related to the 'minimum discrete area.'

Mild shearing stresses applied to the ointment, such as extrusion from its tube or gently spreading onto skin, results in coalescence of the oily droplets into lagoons (Fig. 1a). Typical analyses of the separated liquid revealed as much as 90% w/w of labelled content of the drug in a given sample. 'New' Betnovate® ointment obtained since January 1982 no longer contains globules of this oily liquid nor does it separate upon shear into steroid-enriched lagoons of propylene glycol and relatively drug-free base. Dark-field polarized light microscopy shows the new formulation to contain liquid crystalline complexes which, with their 'Maltese cross' appearance are probably spherulites (Fig. 1b). The degree of homogeneity of drug distribution was appreciably better than the 'old' formulation. When tested at the 25 mg scale by the same analytical procedure, coefficients of variation between samples of 1·78% and 1·54% were obtained for two batches of ten samples. The differences in uniformity between the 'old' and 'new' are exacerbated on dilution with the recommended diluent, white soft paraffin. We found that distribution of drug in new Betnovate® diluted extemporaneously was acceptable (C% = 3·76 and 4·24), when ten 100 mg samples were tested, whereas the same test applied to the old formula Betnovate® (similarly diluted) exhibited huge sample variations (C% = 19·6). It is not clear which material Ryatt *et al.* (1982) used but since their paper was accepted in October 1981 it is probable that they worked with the 'old' formulation.

Although it is not easy to compare the stability data of Ryatt *et al.* (1982) and Yip and Li Wan Po (1979) due to the different dilution levels studied, storage temperatures and the known (but not described) variability of emulsifying ointment, it would seem that Ryatt's group have obtained lower rates of drug conversion than Yip. Our stability data for 1 in 4 dilutions of 'old' Betnovate Ointment in emulsifying ointment B.P. (Macarthy) more closely related to Yip's findings with the half life for betamethasone-17-valerate conversion of 1·3–1·8 h at 20°C. However, upon repeating the work using the 'new' formulation of Betnovate® ointment diluted in similar emulsifying ointment, considerably slower rates of interconversion have been obtained. More than 90% w/w of the labelled content of betamethasone-17-valerate in a dilution was maintained for 8 weeks at room temperature.

Smith (1982) has shown that a large number of prescriptions are issued for extemporaneous steroid

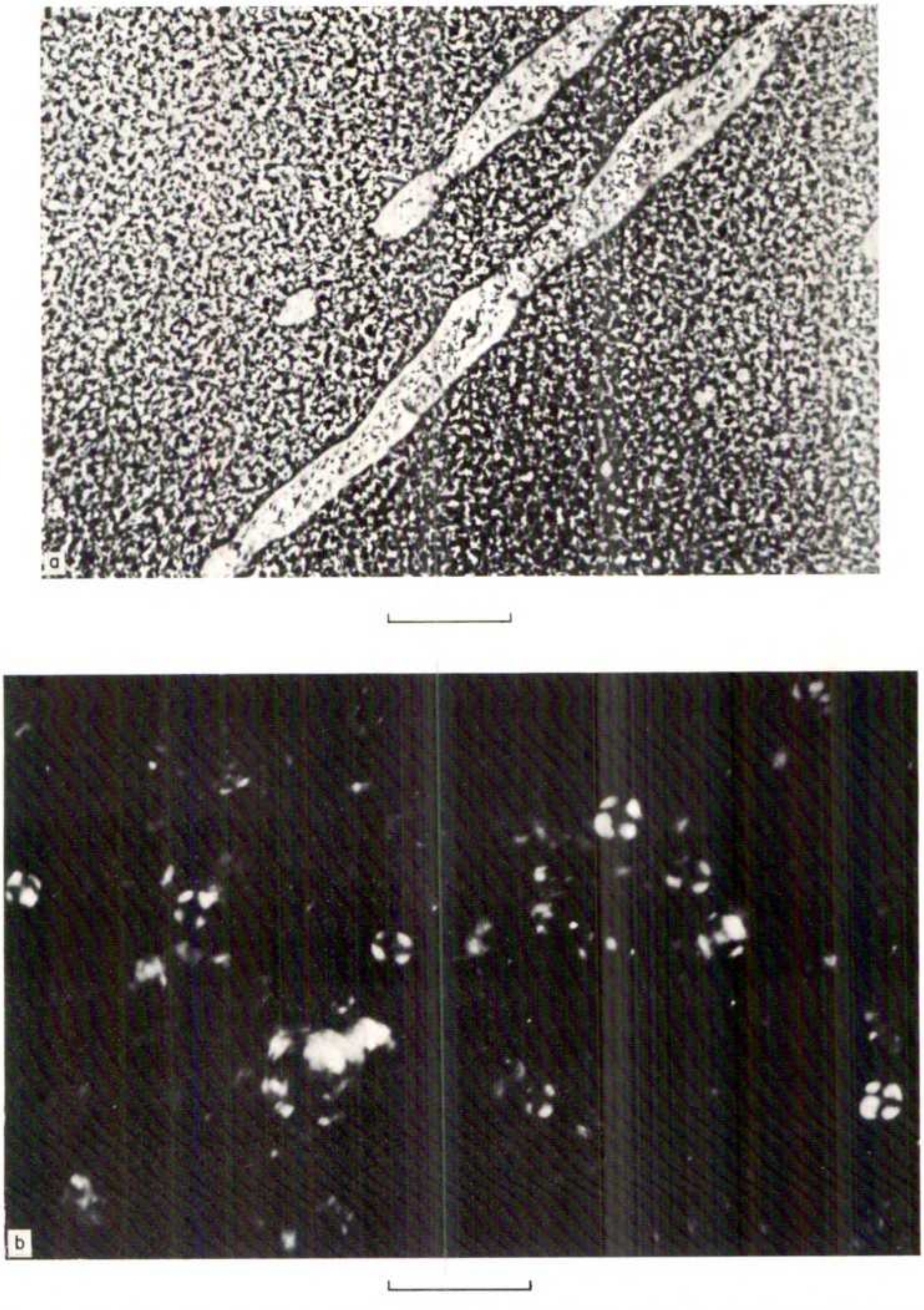

FIGURE. 1. (a) phase-contrast photomicrograph of 'old' Betnovate® ointment after gentle rubbing on microscope slide showing lagoons of steroid-rich oily liquid. (b) photomicrograph of 'new' Betnovate® ointment viewed through crossed polars and dark-field illumination showing liquid crystalline complexes. Bar lines = 100 microns.

dilutions in both recommended and unrecommended bases. Each prescription should be justified by studies of stability, quality and efficacy

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YIP, Y.W. & LI WAN PO, A. (1979) The stability of betamethasone-17-valerate in semi solid bases. *Journal of Pharmacy and Pharmacology*, **31**, 400.

Reply

SIR: The formulation of Betnovate® ointment was changed for two reasons. First, to improve the characteristics of the product and second, to have the same formulation available in the U.K. as had been available for several years in overseas markets. Clinical trials had not shown any significant difference between the two preparations.

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The stability and blanching efficiency of betamethasone-17-valerate in emulsifying ointment

SIR, In their recent paper (*British Journal of Dermatology* 1982, **107**, 71), Ryatt *et al.* have raised some important points which we view with some concern.

First, they suggest that the use of 'stable' dilutions of Betnovate® ointment may be as efficacious as the undiluted preparation and yet is likely to cause fewer side-effects. This may be true regarding systemic side-effects, but if the assumption that clinical efficacy can be predicted from vasoconstriction data is correct, it would be prudent to assume that local side-effect potential follows the same relationship until proven otherwise.

Second, encouragement to use an unstable formulation, the potency of which would depend on storage conditions and the time of application relative to preparation must be deprecated. According to their data, 20 h after dilution the concentration of betamethasone-17-valerate (half life = 4 h) would be equivalent to a 1 in 32 dilution, shown by them to have a blanching capability similar to emulsifying ointment. Such a rapid decay in activity can hardly be regarded as an appropriate method of weaning the patient off topical corticosteroids.

Third, although it is claimed by the authors that the dilution of Betnovate® ointment in emulsifying ointment is widely practised in the U.K., it should be noted that the diluent recommended for Betnovate® ointment is soft white paraffin.

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J.M.Kirsch
J.R.Gibson
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Reply

Sir, We read with interest the points that Kirsch *et al.* raise. In our support we emphasize first the broad consensus about the close relationship of:

(1) the blanching potential and clinical efficacy,
(2) the blanching potential and the local and systemic side-effects of steroids, the latter depending partly on the chemical structure and partly on the amount of steroid used.

Betnovate® ointment, diluted 1:4 in Unguentum Merck®, has already been shown to be stable for up to 9 months and we have shown that dilutions of Betnovate® ointment, up to 1:16, possess blanching potential equipotent with that of the undiluted parent compound. Surely therefore the inference, unproven thus far, must be that both topical and systemic side-effects from these preparations are likely to decrease, as less of the active compound has been used.

Secondly, we have nowhere encouraged the use of an unstable preparation. Indeed, the primary aim of the paper was to demonstrate the instability of Betnovate® ointment in emulsifying ointment. Though the makers (Glaxo Laboratories Ltd.) recommend white soft paraffin as the diluent of choice for Betnovate® ointment, emulsifying ointment enjoys a high degree of popularity not only with dermatologists but also with patients up and down the country. Although 17-valerate, the active constitutent of Betnovate® ointment diluted 1:4 in emulsifying ointment, virtually disappears within 24 h, 21-valerate and free betamethasone alcohol remain, the free alcohol being the dominant compound at 4 weeks. Significant blanching potential and, therefore, potential clinical usefulness remains at 2 weeks, though at 3 weeks this is almost similar to that of the emulsifying ointment. This, to some extent, has been borne out by the fact that we have had no rebound crises in patients with acute/sub-acute dermatoses treated with the above preparations.

The General Infirmary,
Leeds

K.S.Ryatt
J.W.Feather
A.Mehta
J.B.Dawson
J.A.Cotterill
R.Swallow

British Journal of Dermatology (1983) **108**, 253–254.

Book Reviews

Hair Transplantation for the Treatment of Male Pattern Baldness. CHARLES P.VALLIS (1982) Springfield: Thomas. P. xvi 593. Price U.S. $69.75.

The principal author of this comprehensive text is instructor in plastic surgery and dermatology at Tufts University of Boston. Dr Vallis in his preface writes, 'Many unscrupulous opportunists capitalized on this human concern for hair. Hair clinics promising unrealistic results sprung up all over the country. Many untrained physicians primarily interested in monetary benefits attempted hair transplantation, and they were often repaid for their inexperience with unhappy patients and malpractice suits.' Unfortunately, hair transplantation has suffered a similar fate in Britain and in many other countries, and is all too often carried out by the unqualified and unskilled. It is not surprising that many plastic surgeons have been deterred by the association of hair transplants with charlatanism. Dr Vallis has performed a useful service in making more widely known the various surgical procedures by which plastic surgery can offer real cosmetic benefit in some cases of androgenetic alopecia and in some cicatricial alopecias.

The use of punch grafts is by far the most widely used technique in androgenetic alopecia. It was pioneered by Shoji Okuda of Japan in 1939, but reached the western world only after Orentreich's important investigations, a report of which was published in 1959. Vallis gives a detailed account of the techniques, the indications and contra-indications and the possible complications. He describes as well as the punch grafts, the technique of the strip graft of which he has also had much experience. Coiffeman of Bogota contributes an account of square scalp grafts, Ray Elliot of Albany N.Y. describes the use of lateral scalp flaps, and Guy and Bernard Blanchard of Montreal write of their experience of a so-called 'hairy scalp lift,' a technique which involves resecting areas of bald scalp.

This is a useful book, well produced and profusely illustrated and it is to be hoped it will encourage British and other plastic surgeons who have hesitated to become involved in a field in which so many unfortunate men have been exploited. Plastic surgery in the hands of a skilled surgeon has much to offer a carefully selected minority of patients with androgenetic alopecia.

A.J.ROOK

Physics in Physiotherapy—Conference Report Series No. 35. Edited by M.F.DOCKER (1982) The Hospital Physicists' Association: 47 Belgrave Square, London SW1X 8QX. Pp. 42. Price £4.50.

This small monograph is based on a series of nine papers given at a joint meeting of The Hospital Physicists' Association and the Chartered Society of Physiotherapists in May 1980. It is a slim volume of only forty-two pages, and covers, in outline form, the rationale and use of various treatment procedures such as UV irradiation, infra-red and microwave, diathermy and ultra-sound.

Many of the authors acknowledge the lack of objective measurements, so far as response is concerned, in many of the treatments discussed but in general the text is clear and concise and there is a particularly good section on UV irradiation. The book serves as a useful introduction to the theory and practice of the various electrophysical techniques that are now being commonly used in physiotherapy departments. However, as one of the contributors suggested, physiotherapists are going to have to become much more scientific in their evaluations—that something should just appear to work is no longer good enough. This small book is perhaps a useful first step.

JOHN D.WILKINSON

Optoelectronics in Medicine: Proceedings of the 5th International Congress, Laser 81. Edited by W. WAIDELICH (1982) Heidelberg: Springer. Pp. 239. Price DM 62.

Lasers of all types are being used in a variety of clinical and research areas with improving efficacy. These proceedings are one part (the other being Optoelectronics in Engineering) which together cover the Laser 81 Optoelectronics Congress, the most recent in a biennial series, held in Munich. The Congress aims to promote the rapid exchange and discussion of ideas and findings; the speed with which the proceedings have become available is commendable. This very alacrity causes familiar problems—the number of typographical and grammatical errors is only just excusable.

Most workers in the field will find something here to stimulate or help them. Many of the technical papers, whilst describing techniques in an early stage of refinement, will give others the opportunity to evaluate and adapt, admirably fulfilling the Congress' aims. There are disappointingly few clinically orientated papers and the anecdotal nature of some vitiates their usefulness, as does the failure to give references to all exemplified work. However, most are up to standard and show that a great deal of thought and expertise are being devoted to the clinical applications of laser technology.

With the exception of the introduction none of the papers come from the United Kingdom or the United States. It would be nice to see work from these countries included in the next International Congress Proceedings. This is a useful book which should be rapidly assimilated by all those with an interest in laser radiation.

N.P.J. WALKER

Skin Surgery, Fifth Edition. Edited by E. EPSTEIN and E. EPSTEIN, JR. (1982) Springfield: Thomas. Pp. 1241, 2 Vols. Price U.S. $215.00

The previous edition of this book was reviewed in this journal in 1977. The growth of the new 5th edition has paralleled the growth in dermatological surgery as a specialty. There are now 1241 pages in two volumes, compared with 839 in one volume, eighty-three authors compared with forty-three, but still only one from outside North America. Virtually all the techniques that one has heard of, together with a few that one has not, are well assessed, although lasers are still somewhat left out in the cold.

Whatever views are held on the role of the dermatologist in undertaking these procedures, this book has established itself quite rightly as an invaluable standard text, even if only to show what can be done elsewhere. The book is written to suit the dermatologist, the dermatologist with a leaning towards plastic surgery and also the surgeon or oncologist without dermatological training.

The editors firmly state that their policy is not to produce a closely integrated account reflecting essentially their own views. Each author is given carte blanche to air his own views and it is left to the reader to shop around and decide which is the best way to deal with a particular clinical situation. The skills of the operators available are as yet more important then the techniques themselves.

This new edition is timely and welcome.

R.H. CHAMPION

British Journal of Dermatology (1983) **108,** 255–256.

News and Notices

Hair and Aesthetic Medicine, Rome, March 1983

An international Symposium on 'Hair and Aesthetic Medicine' will be held in Rome on 24–26 March 1983, promoted by the International Union of Aesthetic Medicine. For further information, contact Dr L.Celleno, Foundation for Research in Dermatology, Largo A.Gemelli 8, 00168 Rome, Italy.

Progress in Dermatological Diagnosis and Treatment, Brighton, April 1983

A Symposium entitled 'Progress in Dermatological Diagnosis and Treatment' will take place from 14–16 April 1983 at the Hotel Metropole, Brighton, in conjunction with a Meeting of the Dowling Club, with a Clinical Meeting at Brighton General Hospital on 16 April. Further details from Dr Patrick Hall-Smith, Brighton General Hospital, Brighton.

Société de Recherche Dermatologique

At the IVe Congrès de Recherche Dermatologique (Toulouse, France, 30 Sept–2 Oct 1982) the creation of the Société de Recherche Dermatologique was announced.

The object of the Society is the promotion of scientific research into the skin and skin diseases. While the Society is mainly addressed to researchers in French-speaking countries, one of its aims is to foster international exchange and collaboration.

The Committee is composed of President, A. Claudy; Secretary, D.Schmitt; Treasurer, J.P.Ortonne; Officers, J.L.Bonafé, L.Dubertret, J.J.Guilhou, Y.Jacques, G.Orth.

Interested persons are invited to contact the Secretary: Clinique Dermatologique (Pavillon R), Hôpital Edouard Herriot, 69374 Lyon Cédex 2, France.

International Society of Tropical Dermatology, Amsterdam, June, 1983

The International Society of Tropical Dermatology will hold a regional meeting in Amsterdam, The Netherlands, from 2–4 June 1983. For further information contact Professor E. van Dijk, Department of Dermatology, Free University Hospital, de Boelelaan 1117, 1007 MB Amsterdam, The Netherlands.

Dermatopathology Foundation Course, Atlantic City, June 1983

The eleventh annual Dermatopathology Foundation course in gross and microscopic pathology of the skin will be held in Atlantic City, New Jersey, on 13–17 June 1983. For further information contact: Dermatopathology Foundation, P.O. Box 377, Canton, MA 02021, U.S.A.

Topics on Topicals, Cardiff, February, 1983

An intensive seminar on topical therapy will be held in Cardiff on 17 and 18 February 1983. It is intended that the course will be of interest to clinical pharmacologists, dermatologists, cosmetic scientists, chemists and pharmacologists in industry. The emphasis will be on advances in formulation for topical therapy and on the problems of penetration and stability of topical preparations. For further details apply to: Professor R.Marks, Department of Medicine, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XN.

Dermatopathology Workshop, Worcester, Massachusetts, July 1983

The sixth annual Dermatopathology Workshop will be held on 28 and 29 July 1983 at the university of Massachusetts Medical School, Worcester, Massachusetts, U.S.A.

The Workshop will include the study of 100 unknown microsope slides of inflammatory dermatoses and skin tumours. The faculty members include Drs J.Bhawan, Wilma F.Bergfeld and M.Taylor. For further information contact Jag Bhawan, M.D. (Course Director, Univ. Mass. Medical School, Worcester, Massachusetts, 01605 U.S.A. Telephone (617) 856–2212).

Advanced Course in Medical Mycology, London, April 1983

The Institute of Dermatology is holding a course in medical mycology, aimed principally at dermatologists and dermatologists in training, on 14–15 April 1983. The topics to be considered will include the clinical and laboratory aspects of superficial mycoses, the immunology of fungal infection and other aspects of mycology, including histopathology, fungal allergy and therapeutics. The course fee is £50. Further details from Dr R.J. Hay, Institute of Dermatology, St John's Hospital for Diseases of the Skin, Lisle Street, London WC2H 7BJ. The closing date for receipt of applications is 28 February 1983.

Cutaneous Oncology Conference, The Hague, September 1983

An International Conference on Cutaneous Oncology will be held at the Hague, The Netherlands, on 12–16 September 1983. Topics relating to skin cancer and lymphoma will be discussed in lectures, workshops and seminars, with free communications and poster sessions. For further information contact: Richard L. Dobson M.D., Department of Dermatology, Medical University of South Carolina, Charleston, South Carolina, 29425 U.S.A.

National Congress of the Italian Dermatology Society, Perugia, May 1983

The 60th National Congress of the Italian Society of Dermatology and Venereology will be held in Perugia on 12–14 May 1983. The closing dates for Abstracts and Posters is 28 February 1983. Further details from Congress Secretary, Italian Society of Dermatology, Clinica Dermatologica Universita, Policlinico, 06100 Perugia, Italy.

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Full information is available from:
Imperial Chemical Industries PLC,
Pharmaceuticals Division, Alderley House,
Alderley Park, Macclesfield, Cheshire SK10 4TF

Diseases of the Nails and their Clinical Management

Edited by **R. Baran** MD, *Head of Department of Dermatology, General Hospital, Cannes*, and **R.P.R. Dawber,** MA, MB, ChB, FRCP, *Consultant Dermatologist, Oxford Hospitals and Clinical Lecturer in Dermatology, University of Oxford*

This important title will be an essential reference for all clinicians involved in the diagnosis and management of diseases of the nails. It presents a comprehensive account of the structure and function of the nails, and of the clinical and therapeutic aspects of all nail disease, including surgical treatment where appropriate. Chapters have been contributed by a team of authors of international renown and the book is lavishly illustrated with colour plates to assist in diagnosis.

Contents: Embryology, anatomy and physiology *R.P.R. Dawber;* Physical signs *R. Baran;* Investigations *R.P.R. Dawber;* The nail in childhood and old age *R. Baran;* Fungal diseases *R.J. Hay;* The nail in dermatological disease *R. Baran;* The nail in systemic disease *D. Fenton and J.D. Wilkinson;* Occupational abnormalities and contact dermatitis *R.J.G. Rycroft;* Genetic abnormalities *L. Juhlin;* Surgical treatments (and skin tumours) *H. Bureau, E. Haneke and R. Baran;* Cosmetics *E. Brauer.*

Summer 1983. 500 pages, 300 illustrations (200 in colour). About £48.00

Blackwell Scientific Publications

Essentials of Industrial Dermatology

Edited by **W.A.D. Griffiths** MD, MRCP
Consultant Dermatologist, St. John's Hospital for Diseases of the Skin and Senior Lecturer, Institute of Dermatology, London

and **D.S. Wilkinson** FRCP
Consultant Dermatologist, Wycombe General Hospital

This compact handbook provides guidelines and sets out basic principles in the field of industrial dermatology for doctors working as full- or part-time factory doctors, who may have little previous dermatological training. It will also appeal to doctors working in public or community health, particularly in the developing countries where dermatological problems are becoming increasingly apparent. The text includes line drawings and tables for easy reference.

Contents: A national problem *W.A.D. Griffiths;* Industrial dermatitis today and its prevention *D. Burrows;* Constitutional skin diseases in industry *J. Cotterill;* Investigation of industrial dermatitis *C. Hindson;* Soluble oils *R. Rycroft;* Hand eczema in industry and the home *R. Church;* Chemical acne *D. Williamson;* Principles in dermatitis litigation *R. Bursell;* Conclusions and key points *D.S. Wilkinson & W.A.D. Griffiths.*

Spring 1983. 150 pages, 20 illustrations. About £10.00

Blackwell Scientific Publications

NOTICE TO CONTRIBUTORS

The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advances in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

Papers accepted become the copyright of the Journal. This Journal is covered by *Current Contents, ISI/BIOMED, Science Citation Index and ASCA.*

Manuscripts should be sent to the Editor, Dr J.L.Burton, Dermatology Department, Bristol Royal Infirmary, Bristol BS2 8HW. They should be typewritten on one side of the paper only, with a wide margin, be double spaced, and bear the title of the paper, name and address of each author, together with the name of the hospital laboratory or institution where the work has been carried out. The name and full postal address of the author who will be responsible for reading the proofs should be given on the first page. A duplicate copy, complete with figures, tables and references, should be sent, since in many cases two referees are consulted. The author should keep a carbon copy of his manuscript. The Editorial Board reserves the right to make literary corrections.

Abbreviations and units. Full names with uncommon abbreviations must be given with the first mention: new abbreviations should be coined only for unwieldly names and should not be used at all unless the names occur frequently. In the title unusual abbreviations should be identified, in the introduction and discussion they should be used sparingly. Abbreviations of units should conform with those shown below.

gram(s)	g	minute(s)	min	molar	mol/l
kilogram(s)	kg	centimetre(s)	cm	millilitre(s)	ml
milligram(s) (10^{-3}g)	mg	second(s)	s	gravitational acceleration	*g*
microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm³	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
hour(s)	h	milliequivalent	mmol		

These and other abbreviations and symbols should follow the recommendations of: *Units, Symbols and Abbreviations,* A Guide for Biological and Medical Editors and Authors (1971) The Royal Society of Medicine, London.

Illustrations should be referred to in the text as 'Figs', and be given Arabic numbers, and should be marked on the back with the name(s) of the author(s) and the title of the paper. Where there is any possible doubt as to the orientation of an illustration, the top should be marked with an arrow. Each figure should bear a reference number corresponding to a similar number in the text. Photographs and photomicrographs should be unmounted glossy prints and should not be retouched. Colour illustrations will be accepted when found necessary by the Editor, although the author will be expected to bear the cost. Diagrams should be on separate sheets; they should be drawn with black ink on white paper on feint-ruled graph paper and should be about twice the size of the final reproduction. Lines should be of sufficient thickness to stand reduction. Each illustration should be accompanied by a legend clearly describing it; these legends should be grouped on a separate sheet of paper.

There should be as few tables as possible and these should include only essential data; they should be typewritten on separate sheets and should be given Arabic numbers.

References. Only papers closely related to the author's work should be referred to; exhaustive lists should be avoided. References should be made by giving the author's surname, with the year of publication in parentheses. When reference is made to a work by three authors, all names should be given when cited for the first time and thereafter only the first name adding *et al.*, e.g. Blackett *et al.* (1952); for four or more authors, the first name followed by *et al.* should be used on all occasions. If several papers by the same author and from the same year are cited, a, b, c, etc., should be put after the year of publication, e.g. Blackett *et al.* (1952a) or Blackett *et al.* (1952a,b). All references should be brought together at the end of the paper in alphabetical order. References to articles and papers should mention: (1) name(s) followed by the initials of the author(s); (2) year of publication in parentheses; (3) title of paper; (4) title of journal in full; (5) volume; (6) number of the first page of the article. *Thus*: CUNLIFFE, W.J. & SHUSTER, S. (1969) The rate of sebum excretion in man. *British Journal of Dermatology*, **81**, 697. References to books and monographs should include: (1) author(s) or editor(s); (2) year of publication; (3) title; (4) edition, volume etc.; (5) page referred to; (6) publisher; (7) place. *Thus*: BEARE, J.M. & WILSON JONES, E. (1972) Necrobiotic disorders. In: *Textbook of Dermatology* (Ed. by A.J.Rook, D.S.Wilkinson and F.J.Ebling), 2nd edn, Vol. 2, p. 1066. Blackwell Scientific Publications, Oxford.

Page proofs will be submitted without the original typescript to the author for proof-correction (see under 'manuscripts') and should be returned to the Editor within three days. Major alterations from the text cannot be accepted.

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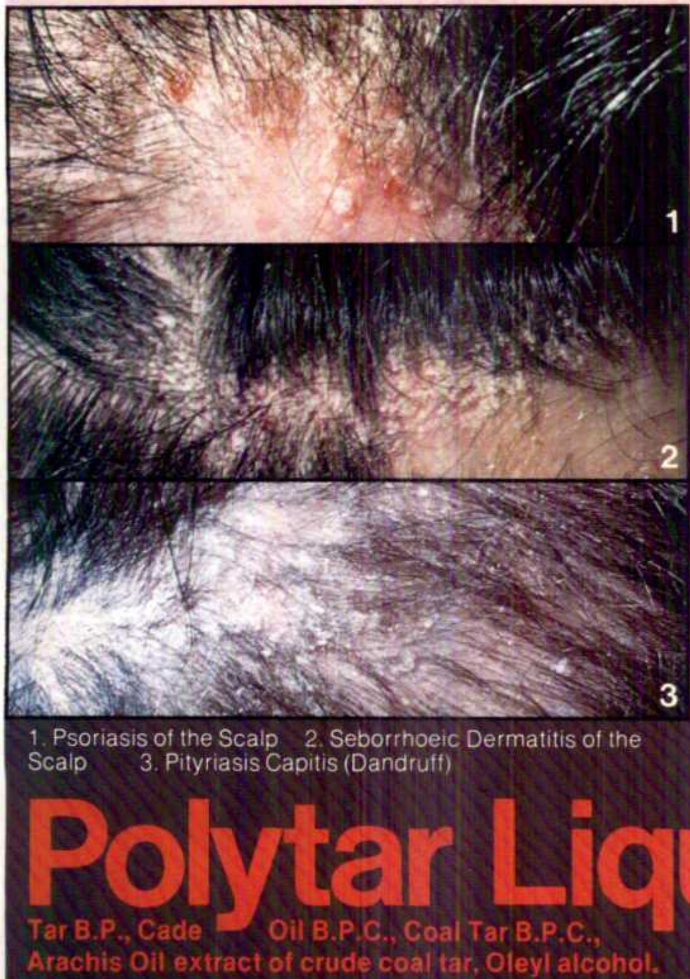

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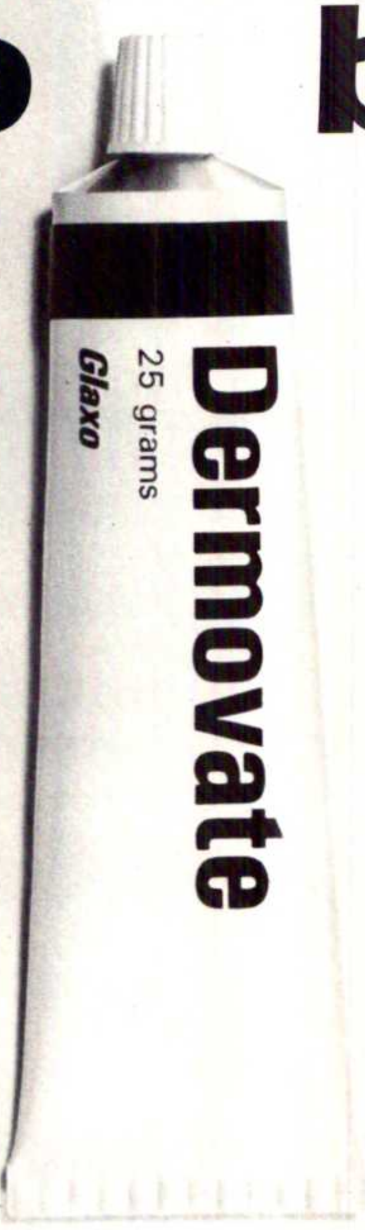

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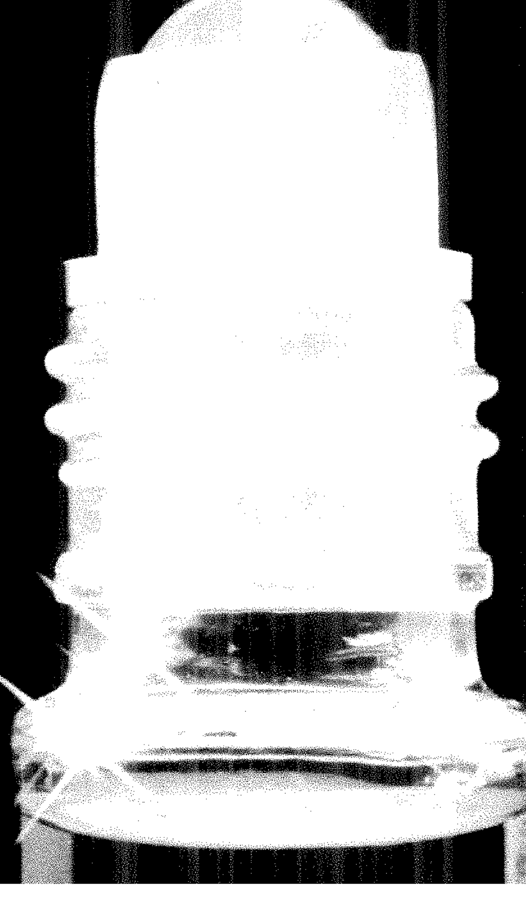

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British Journal of Dermatology (1983) **108**, 257–262.

Clinical and Laboratory Investigations

Immunohistochemical demonstration of proteoglycans in the skin of patients with systemic sclerosis

M.KITABATAKE, H.ISHIKAWA AND H.MAEDA

Department of Dermatology, School of Medicine, Gunma University, Maebashi, Japan

Accepted for publication 9 July 1982

SUMMARY

Skin proteoglycan was demonstrated by an immunofluorescent technique using an antibody against bovine cartilage proteoglycan, after the cross-reactivity of human proteoglycan with the antiserum had been confirmed. Normal skin exhibited specific fluorescence mainly in the blood vessels as well as in the subepidermal area. The clinically uninvolved skin of systemic sclerosis (SS) revealed no features different from those of normal skin. However, the vascular proteoglycan deposition of early systemic sclerosis was later replaced by deposition between the collagen fibres, which appeared to progress centrifugally in parallel to the increase in the skin sclerosis, suggesting a vascular initiation of the skin lesion. Sclerotic skin was characterized by random deposition between the collagen fibres. Immunoelectron microscopic studies suggested that the random proteoglycan deposition reflected uncontrolled local accumulation of proteoglycan in the interfibrillar matrix around irregularly arranged collagen fibrils.

Proteoglycan (PG) or PG monomer is composed of glycosaminoglycan (GAG) chains bound to a specific protein called 'core protein'. The PG of cartilage is connected to a long chain of hyaluronic acid stabilized by link proteins (PG aggregates) (Hardingham & Muir, 1972; Hascall & Heinegård, 1974a, b). There is evidence that PG may also exist in the skin (Miyamoto & Nagase, 1980). Using an electron microscope, Maeda, Ishikawa & Ohta (1981) observed a structure consisting of hyaluronic acid bound to PGs in the matrix of the skin. This structure resembled the PG aggregate observed by Rosenberg, Hellmann & Kleinschmidt (1975) in bovine cartilage.

Systemic sclerosis (SS) is a connective tissue disease characterized by sclerotic fibrosis. The essential change inducing the skin sclerosis remains to be clarified. Increased PAS staining of the involved skin was first noted by Braun-Falco (1957). Biochemical analyses by Neldner,

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Correspondence: Dr H. Ishikawa, Department of Dermatology, School of Medicine, Gunma University, Showa-Machi, Maebashi, Japan.

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Jones & Winkelmann (1966), Black, Bottoms & Shuster (1970) and Blumenkrantz, Ullman & Asboe-Hansen (1976) have disclosed diminished collagen or hydroxyproline content in the sclerotic skin, indicating either a comparative increase in non-collagenous components or a different chemical composition of the collagen in SS. Our previous electron microscopic investigation revealed an increase in Ruthenium red-positive granules around the collagen fibrils. We also induced skin sclerosis experimentally with a heparin sulphate-like GAG isolated from the urine of patients with SS (Ishikawa *et al.*, 1975, 1978). These findings indicate the importance of the GAG or PGs in the pathogenesis of scleroderma.

In the present study, we have attempted to demonstrate skin PG immunohistochemically in SS, and to follow any alteration as the disease progresses.

METHODS

Skin specimens were obtained from the extensor aspect of the forearm and the back of fifteen adult patients with SS (thirteen female) and were classified into three stages: (1) clinically uninvolved (one specimen from the forearm, four specimens from the back); (2) moderately involved (seven specimens from the forearm, four specimens from the back); (3) markedly involved (sclerotic) (seven specimens from the forearm, two specimens from the back), as described previously (Ishikawa & Horiuchi, 1975). As controls, we used site-matched normal skin from adult females (four specimens from the forearm, two specimens from the back) as well as lesional skin from four patients with circumscribed scleroderma (morphoea), and skin from two patients with scleroedema of Buschke and four patients with keloid. In addition, the lesional skin of a patient with lichen myxoedematosus was used as a negative control, because faulty formation of PG in circumscribed myxoedema has been described in a previous report (Maeda *et al.*, 1981). The specimens were kept at −80 °C until use.

Demonstration of skin PG

PG was isolated from bovine nasal cartilage by the method of Heinegård (1972). In summary, cartilage slices were extracted with 4 M guanidine hydrochloride in 0·05 M sodium acetate, pH 5·8, containing protease inhibitors, and then were dialysed against 0·05 M sodium acetate with the protease inhibitors. PG(A_1) was isolated by CsCl density gradient centrifugation. A portion of the sample was further centrifuged in CsCl and 4 M guanidine-HCl to separate PG monomers (A_1-D_1) from link protein. The PG preparations were identified by chemical determinations of compositions and SDS polyacrylamide gel electrophoresis of PG, as well as by cellulose acetate membrane electrophoresis of the GAG component. Chemical analysis of our PG sample corresponded to that of Heinegård (1972). GAG was composed exclusively of chondroitin sulphate.

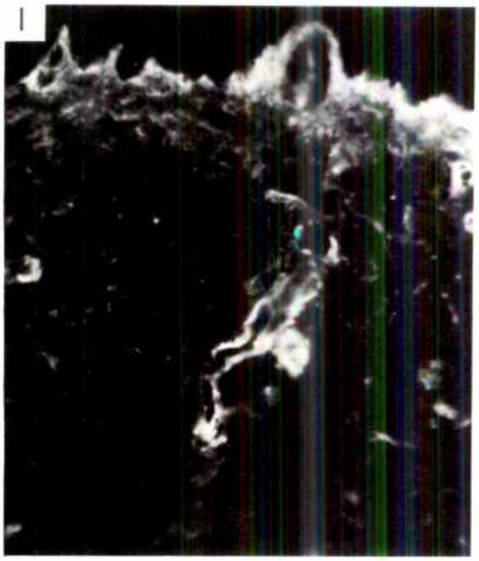
An antiserum to bovine cartilage PG was prepared according to a modified procedure of Wieslander & Heinegård (1979). Japanese white rabbits weighing about 2·5 kg were subcutaneously injected at many sites with 10 mg of PG(A_1). The antigen, dissolved in 1 ml of 0·9% NaCl, was mixed with the same volume of Freund's complete adjuvant. Booster doses with 3 mg in 0·6 ml of 0·09% NaCl, mixed with an equal volume of Freund's incomplete adjuvant, were given 2 weeks later and then every second week until the antibody titre became constant. Antisera obtained from the ear venule were checked every 2 weeks against PG (A_1), PG monomer and link protein by passive haemagglutination test (Daniel, Weyand & Stavistsky, 1963), using formalin-fixed human red cells (blood group O, Rh positive) with 10 μg antigen/10^9 cells. The maximum titre was obtained 5 months after the first immunization. The antiserum reacted with A_1 or A_1-D_1 up to a dilution of 1:6400. The antiserum reacted to a lesser extent with link protein. Hyaluronic acid (Seikagaku Kogyo, Japan), fibronectin (from human serum, Sigma, USA) and skin collagen (type I, III) failed to react with the antiserum. By the immunodiffusion technique, no precipitin line was observed between PG preparations and the antiserum. The antiserum was further purified by an affinity chromatographic technique, using CNBr-activated Sepharose 4B (Pharmacia, Sweden).

Reactivity of human PG with the antiserum against bovine cartilage PG was examined using human cartilage and skin PG. For this purpose, human articular cartilage taken during orthopaedic surgery was used for the preparation of human cartilage PG in a manner similar to that for bovine cartilage PG. Skin PG was isolated from the normal-appearing skin resected at the operation for a malignant melanoma, after the procedure of Miyamoto & Nagase (1980). The GAG of skin PG obtained was composed of dermatan sulphate with a minimal amount of hyaluronic acid and a chondroitinase ABC-resistant GAG. Immunological reaction was examined by passive haemagglutination test. Erythrocytes sensitized to human cartilage or skin PG (10 μg/10^9 cells) reacted with the bovine cartilage PG antiserum diluted 1:3200. For immunofluorescent staining, 6-μm-thick, air-dried, acetone-fixed cryostat sections were first incubated with either crude PG antiserum (dilution 1:80 or 1:160) or purified antibody (300 μg protein per ml in PBS) at 37°C for 30 min. After extensive washing with PBS, they were further incubated with FITC-labelled swine anti-rabbit IgG antiserum (Dakopatts Denmark, F/P ratio 3·5), diluted 1:40, at 37°C for 30 min. The sections were again washed with PBS, embedded in glycerol, and observed with a Zeiss fluorescence microscope.

The specificity of the staining was confirmed by the disappearance of the fluorescence in sections treated with anti-PG antiserum which had been previously adsorbed by human skin PG. As a control, normal rabbit serum instead of the anti-PG antiserum was used. Immunoelectron microscopic demonstration of the skin PG was carried out using an immunoperoxidase technique. Slices separated into 1 mm flakes were first fixed in periodate-lysine-paraformaldehyde (McLean & Nakane, 1974) for 3 h, rinsed in PBS overnight, and incubated in crude rabbit antiserum to bovine cartilage PG (diluted 1:20) for 12 h. Thereafter, they were rinsed in PBS overnight and incubated in horseradish peroxidase (HRP) conjugated swine antiserum to rabbit IgG (Dakopatts, dilution 1:20) for 12 h. After rinsing in PBS, the specimens were fixed in 2% glutaraldehyde in PBS for 1 h, and rinsed again in PBS. After incubation in Graham & Karnovsky medium, they were rinsed in PBS, and post-fixed in 1% OsO_4 for 2 h. The above procedures were carried out at 4°C. After dehydration and embedding in epon, ultra-thin sections were observed under a JEOL JEM T8 electron microscope with and without uranyl acetate-lead staining.

RESULTS

In normal skin, specific fluorescence was seen in the subepidermal zone and in the blood vessels. Furthermore, there was a faint fluorescence between the collagen fibres. Clinically uninvolved skin of SS exhibited no evident alteration from normal (Fig. 1), but moderately involved skin demonstrated disseminated, fine granular PG deposits around the blood vessels. In markedly involved skin, randomly arranged PG deposits of a coarse granular form were scattered between the collagen fibres (Fig. 2), particularly in the lower layer of the corium (Fig. 3). Fluorescence in areas close to the blood vessels and in the subepidermal zone was rarely discernible in sclerotic skin. The lesion of circumscribed scleroderma (morphoea) showed staining features similar to those of SS, although the fluorescence was more intense in the former. On the other hand, the lesional skin of scleroedema of Buschke showed thin linear PG deposition around the collagen fibres (Fig. 3, insert). The keloid demonstrated fine granular PG deposits arranged regularly along the long axis of the collagen fibres. In the circumscribed myxoedema of lichen myxoedematosus, no fluorescence was visible, although the PG-specific fluorescence was observed in the periphery of the lesion between the collagen fibres.



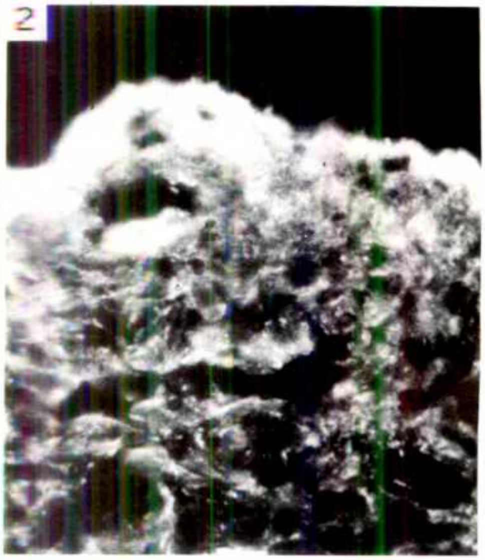


FIGURE 1. Photomicrograph of uninvolved skin of systemic sclerosis stained with anti-bovine cartilage proteoglycan antibody (× 350). This staining pattern is indistinguishable from that in the skin of normal individuals.

FIGURE 2. Photomicrograph of markedly involved (sclerotic) skin showing increased proteoglycan deposits between collagen fibres (× 350).

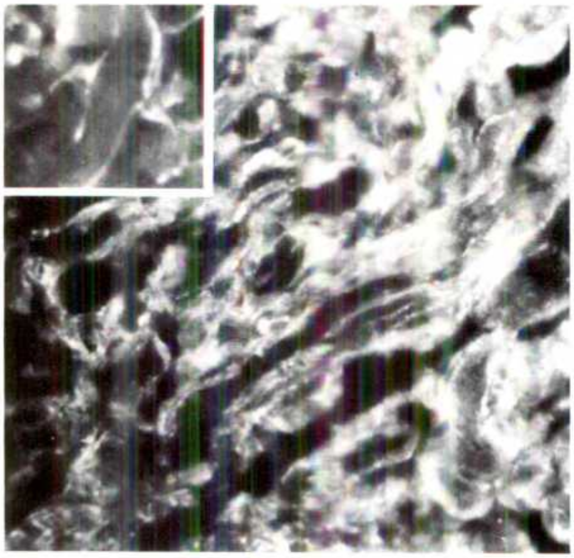


FIGURE 3. Photomicrograph showing random deposits of skin proteoglycan in the lower layer of sclerotic skin (× 350). Insert shows proteoglycan deposits in scleroedema of Buschke (× 350).

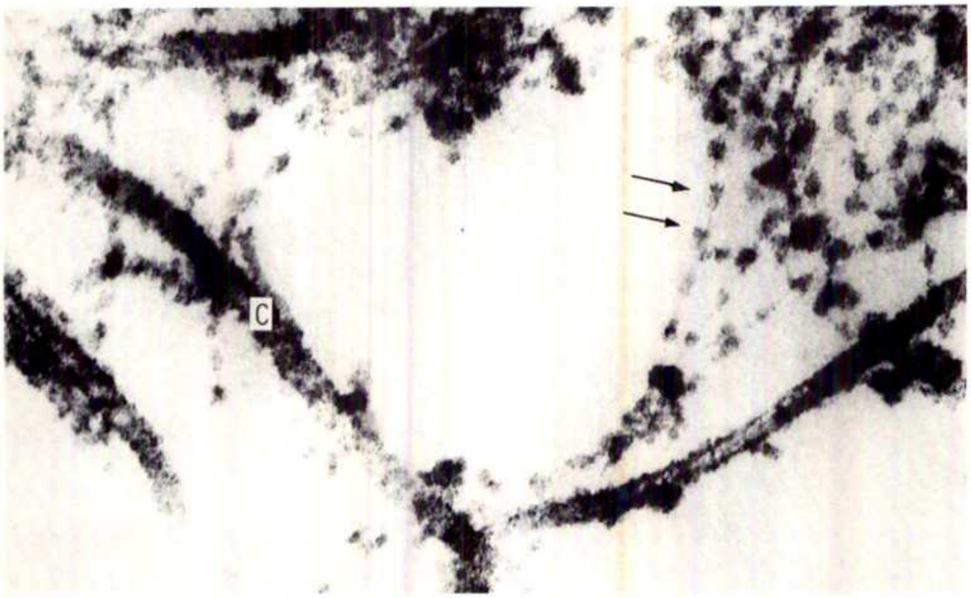


FIGURE 4. Electron micrograph of sclerotic skin, exhibiting immunoperoxidase reaction products (arrows) attached to a thin filament. C = collagen fibril. No contrast staining with uranyl acetate-lead (× 64,000).

HRP-positive granules indicating the ultrastructural localization of PG were abundant in the interfibrillar matrix around loosely-bundled collagen fibrils in the normal and lesional skin of SS. In compact collagen bundles, only the periphery was stained. The reaction products were attached to a thin filament, probably representing hyaluronic acid (Fig. 4). In addition, in the perivascular area of a specimen from the moderately involved skin of SS, a transition from this proteoglycan aggregate-like structure to a cross-banded structure was observed.

DISCUSSION

The fact that we had observed by electron microscopy a PG aggregate-like structure in human skin similar to that of bovine cartilage (Maeda *et al.* 1981) led us to find the skin PG in the present study, using an antibody to bovine cartilage PG. The currently demonstrated ultrastructural features of PG corresponded with those of ruthenium red staining in our previous report, and indicated the presence of PG aggregates. The chemical characterization of PG in the present study remains to be elucidated.

The present investigation showed that PG deposits extended from the vascular area into the surrounding connective tissue with the progress of the skin change in SS. Gay *et al.* (1980) reported an increase in type V collagen, which exists around the blood vessels and in the subepidermal zone, as the initial change of this disease. The present result might provide additional evidence for the vascular initiation of skin lesion in SS. Furthermore, it seems noteworthy that the deposition of PG was random in the sclerotic skin of SS and morphoea. This PG distribution pattern definitely differed from that of either scleroedema of Buschke or keloid. In these diseases, PG deposits were regularly arranged between or around the collagen fibres. Since HRP-positive granules were observed in particular by electron microscopy in the surroundings of loosely-bundled collagen fibrils, random deposition may mean disturbance of collagen aggregation. Recently, Fleischmajer *et al* (1981) described differing collagen synthetic activities of fibroblasts in the skin lesions of SS, depending upon the skin level biopsied. Uncontrolled local accumulation of both PG and collagen, leading to disturbed collagen–PG interaction, might be responsible for the occurrence of skin sclerosis.

ACKNOWLEDGMENTS

This work has been supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture and by Research Grants for Intractable Diseases from the Ministry of Health and Welfare, Japan.

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Immunofluorescence findings in haematoporphyrin-induced keloid

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SUMMARY

A case of haematoporphyrin-induced keloid is described in which immunofluorescence (IF) revealed antibodies eluted from circulating lymphoid cells directed against fibroblasts. The antibodies eluted belonged to the main Ig classes IgD and IgM and they did not bind complement *in vitro*. Their possible activity in stimulating fibroblasts is discussed.

Cheloids result from excessive production of young collagen that follows injury to the skin. For instance, they may occur as a late consequence of intramuscular injection of haematoporphyrin, a drug still in use as an antidepressant agent, which is able to produce phototoxic dermatitis, pigmentation and hypertrichosis.

For a long time, tiny red-brown granules representing residual drug have been known to occur in the dermis at the site of injection (Vilanova & Pinol, 1956). It was suggested that they stimulated fibroblasts in the surrounding skin although such a mechanism is not in keeping with more recent experimental evidence. In fact, fibroblasts cultured in a medium containing porphyrins and exposed to UV radiation have been shown to lose their capacity to proliferate (Schothorst, Suurmond & Ploem, 1977). In addition, their DNA synthesis has been shown to be strongly inhibited (Olmos & Ntuite, 1978).

We report here the immunological findings in a case of haematoporphyrin-induced keloid and propose a pathogenetic mechanism.

CASE REPORT

A woman aged 37 years was treated for 1 month with daily intramuscular injections of haematoporphyrin for depression. After sun-bathing she noticed erythema followed by blistering at the injection sites on her buttocks. Once the blisters had dried up the skin took on a red-brown colour and 4 months later a hard, stellate cheloidal scar was visible (Fig. 1).

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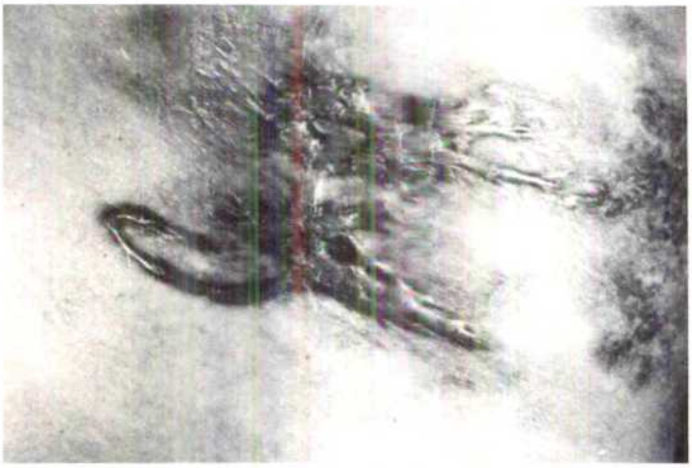

FIGURE 1. Stellate cheloidal scar.

MATERIALS AND METHODS

Immunofluorescence studies

Direct (DIF) and indirect (IIF) immunofluorescence studies were performed on the patient's skin and on guinea-pig lip. In addition, antibodies eluted from circulating lymphoid cells were exposed to various substrates by IIF. They were: skin involved by the keloid, uninvolved skin immediately around the lesion, healthy skin from the contralateral site, and guinea-pig lip.

The specimens were prepared and examined according to the technique described previously by Cormane, Szabo & Hauge (1970).

(i) *DIF studies*. For the demonstration of tissue and cell-bound immunoglobulins (i.e. IgG, IgA, IgM, IgD and IgE) and C3 in the skin specimens, the following conjugates were used: GA Hu IgG/FITC* F/P ratio 1·35, working dilution (wd) 1:60; RA Hu IgA/FITC* F/P ratio 0·8, wd 1:40; GA Hu IgM/FITC* F/P ratio 2·8, wd 1:40; SH Hu IgD/FITC* F/P ratio 2·4 wd 1:40 GA Hu IgE/FITC* F/P ratio 2·1, wd 1:40. HO Hu C_3/FITC† wd 1:60.

(ii) *IIF studies*. The indirect immunofluorescence technique was used on involved and uninvolved skin for the detection of circulating antibodies and antibodies eluted from circulating lymphoid cells (Cormane, Hamerlinck & Nunzi, 1979). Species specificity was verified on guinea-pig lip.

(iii) *In vitro complement fixation tests*. The method described by Jordon, Nordby & Milstein (1961) and detailed by Cormane *et al.* (1979) was used.

A Leitz Orthoplan microscope with a Xenon WBO-751 lamp and a Leitz vertical illuminator were used. FITC fluorescence was detected by using the filter combination BG 38-KP 490 and secondary filter OG 515.

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RESULTS

Direct and indirect immunofluorescence with serum gave negative results in both involved and uninvolved skin. In contrast, indirect immunofluorescence with antibodies eluted for circulating lymphoid cells showed a homogeneous pattern of IgD, directed against fibroblasts in both the involved and uninvolved skin (Fig. 2). In the lesion and in the skin immediately around it IgD and IgM directed against the epidermal nuclei were also found. Such IgD reacted also with epidermal and perimysial nuclei of guinea-pig lip and therefore cannot be considered as species specific. In addition, they did not bind complement *in vitro* using guinea-pig lip as substrate.

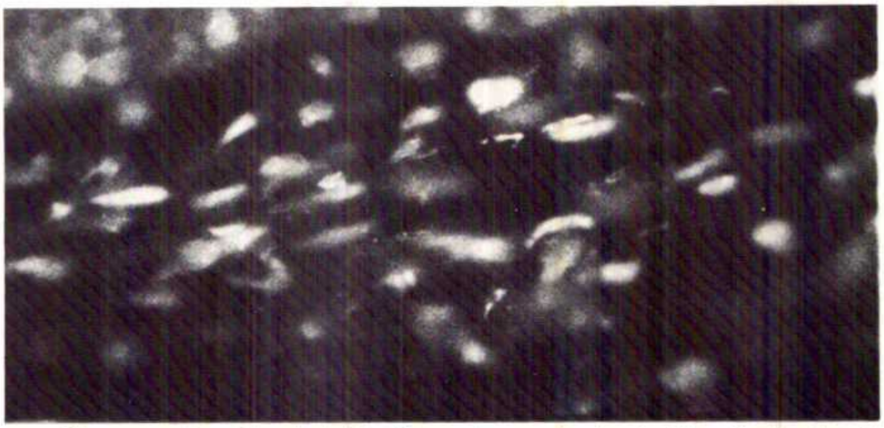

FIGURE 2. Cryostat section of involved skin. The section was incubated with antibodies eluted from circulating lymphoid cells of the patient and subsequently with SH Hu IgD/FITC. Note that IgD present in the eluate is reactive to epidermal cells and fibroblasts in the upper dermis.

DISCUSSION

In our patient, circulating lymphoid cells were shown to bear IgD that reacted *in vitro* with fibroblasts of both involved and uninvolved skin. This finding has been confirmed by Janssen de Limpens & Cormane (1983).

IgD antibodies may be simply an epiphenomenon without functional relevance for the *in situ* production of the disease. Alternatively, one can speculate that the residual haematoporphyrin granules at the injection site, through the concomitant generation of IgD antibodies and the UV action, can enhance the synthesis of collagen locally by fibroblasts. The latter hypothesis, though attractive, would need to be confirmed by *in vitro* experiments.

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Immunohistochemical studies of amyloid P component and fibronectin in erythropoietic protoporphyria

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SUMMARY

Immunofluorescence staining of exposed skin from patients with erythropoietic protoporphyria (EPP) with antibodies to serum amyloid P component (SAP) and to fibronectin produced striking fluorescence of abnormal vascular structures in the upper dermis. An appearance of linear fluorescence along the dermo-epidermal junction with anti-SAP was the result of confluent staining of papillary oxytalan fibres. Amyloid P component (AP) was localized in ultrastructural immunoperoxidase studies to the peripheral (abluminal) regions of thickened dermal vessel walls, the site of maximum concentration of an amorphous matrix containing microfibrillar structures; antibodies to SAP did not bind to leaflets of the reduplicated vascular basal lamina. The characteristic thickening and reduplication of blood vessel walls seen with the electron microscope in EPP therefore involves increased local deposition of both AP and fibronectin.

Examination of skin biopsies from light-exposed areas in erythropoietic protoporphyria (EPP) reveals the characteristic deposition of periodic-acid Schiff (PAS)-positive amorphous hyaline material in and around the walls of upper dermal blood vessels (Peterka, Fusaro & Goltz, 1965; Ryan, 1966; Rimington *et al.*, 1967; Ryan & Madill, 1968; Anton-Lamprecht & Meyer, 1970; Epstein, Tuffanelli & Epstein, 1973). Ultrastructural studies of EPP have shown that the hyaline thickening of vascular walls is the result of multiple concentric reduplication of the vascular basal lamina and the presence of a finely fibrillar material permeating and surrounding the vessel wall (Ryan & Madill, 1968; Anton-Lamprecht & Meyer, 1970; Epstein *et al.*, 1973;

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Nonaka *et al.*, 1975; Gschnait, Wolff & Konrad, 1975; Kint & de Weert, 1978). The formation of the new vascular basement membrane layers involves the deposition of increased amounts of the major structural proteins type IV collagen and laminin which are consituents of normal basement membrane matrices (Wick, Hönigsmann & Timpl, 1979). Amyloid P component (AP), a glycoprotein found in deposits of all forms of amyloid (Cathcart, Skinner & Cohen, 1971; Westermark, Skinner & Cohen, 1975; Shirahama, Skinner & Cohen, 1980), has recently been reported not only to be identical to the normal plasma protein, serum amyloid P component (SAP) (Pepys *et al.*, 1977; Skinner *et al.*, 1980), but also to be an integral consitituent of normal human glomerular basement membrane (Dyck *et al.*, 1980) and of normal elastic fibre microfibrils (Breathnach *et al.*, 1981a,b, 1982a). Fibronectin is a major connective tissue protein which is widely distributed in normal human tissues (Ruoslahti, Engvall & Hayman, 1981) and is also present in vascular and other basement membranes (Stenman & Vaheri, 1978; Fyrand, 1979; Couchman *et al.*, 1979). Aggregated SAP binds fibronectin selectively *in vitro* (de Beer *et al.*, 1981). We have therefore investigated the distribution of AP and of fibronectin in exposed skin of patients with EPP.

METHODS

Skin biopsy material studied

Skin biopsies were obtained from ten patients with EPP in whom the diagnosis had been established on clinical and biochemical criteria, and were taken from the dorsum of the hand overlying the knuckles. Tissue was either snap-frozen immediately for immunofluorescence, or was cut up into blocks and fixed for 10 min at 4°C in 3% w/v paraformaldehyde in phosphate-buffered saline pH 7·4 (PBS) containing 8·5% w/v sucrose for immunoperoxidase studies. The blocks of fixed tissue were washed in two changes of PBS/sucrose at 4°C for 5 h, and were then snap-frozen.

Immunohistochemical studies

Human SAP (Pepys *et al.*, 1977) and fibronectin (Vuento & Vaheri, 1979) were isolated by established techniques. Cryostat sections 4 μm thick of fresh unfixed skin biopsy material were stained with fluorescein-conjugated (FITC) rabbit anti-human SAP and anti-human fibronectin (Breathnach *et al.*, 1981a). Control sections were stained with FITC-anti-SAP and FITC-anti-fibronectin which had been preabsorbed with isolated pure SAP and fibronectin respectively. Cryostat sections 4 μm and 15 μm thick of fixed skin were stained with horseradish peroxidase-conjugated sheep anti-human SAP as previously described (Breathnach *et al.*, 1981b, 1982a). Control sections were incubated with peroxidase-conjugated anti-SAP which had been preabsorbed with isolated pure SAP. The peroxidase activity of bound conjugate was detected histochemically by incubation with 3,3′ diaminobenzidine (BDH Chemicals, Poole, Dorset) and hydrogen peroxide (Graham & Karnovsky, 1966). The 15 μm sections were postfixed in osmium tetroxide and processed for electron microscopy (Breathnach *et al.*, 1981b, 1982a). Ultrathin sections were counterstained with uranyl acetate and lead citrate and were examined in an AEI-801 transmission electron microscope.

RESULTS

Immunofluorescence and light microscopy

Immunofluorescence staining with anti-SAP and with anti-fibronectin antibodies of exposed skin from patients with EPP produced bright specific linear fluorescence of elongated,

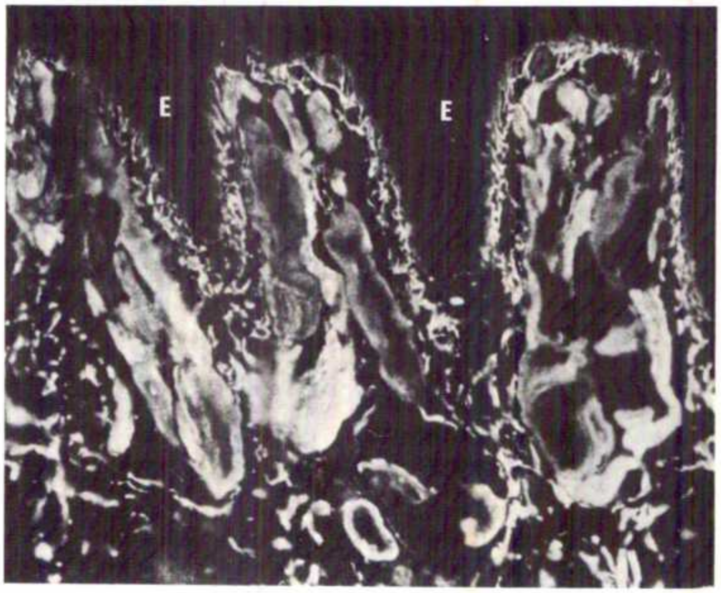

FIGURE 1. Immunofluorescence staining of EPP with anti-SAP. There is bright specific fluorescence of greatly thickened blood vessel walls in the papillary dermis. An appearance of linear BMZ fluorescence is the result of confluent staining of oxytalan fibres. E = epidermis. (× 252).

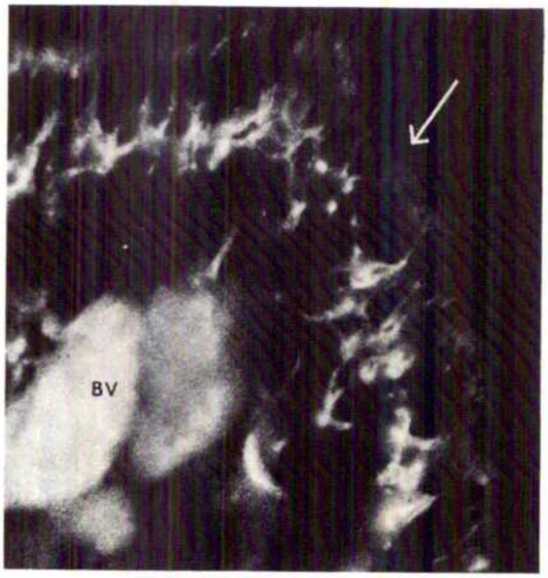

FIGURE 2. Immunofluorescence staining of EPP with anti-SAP. Under higher power, the linear BMZ band is seen as a lower ramifying plexus of oxytalan fibres which connects with a fainter upper line of fluorescence (arrowed). BV = blood vessel. (× 479).

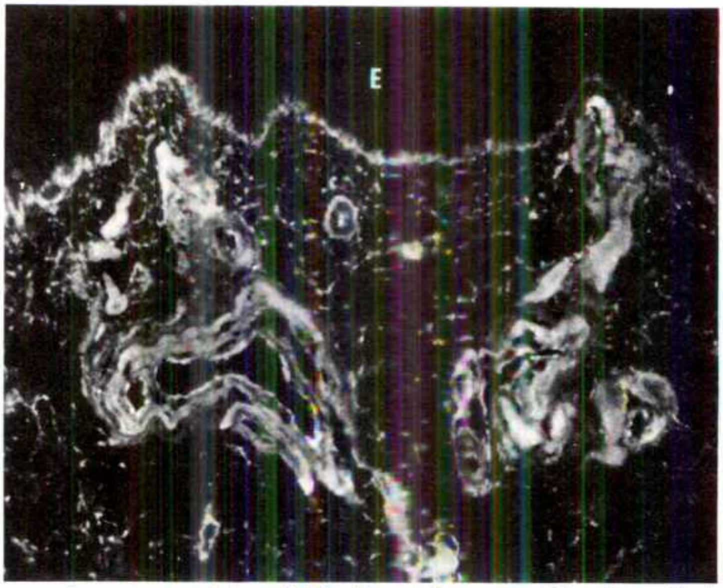

FIGURE 3. Immunofluorescence staining of EPP with anti-fibronectin. There is specific fluorescence of thickened blood vessel walls in the papillary dermis and in a linear band-like pattern along the BMZ. E = epidermis. (× 249).

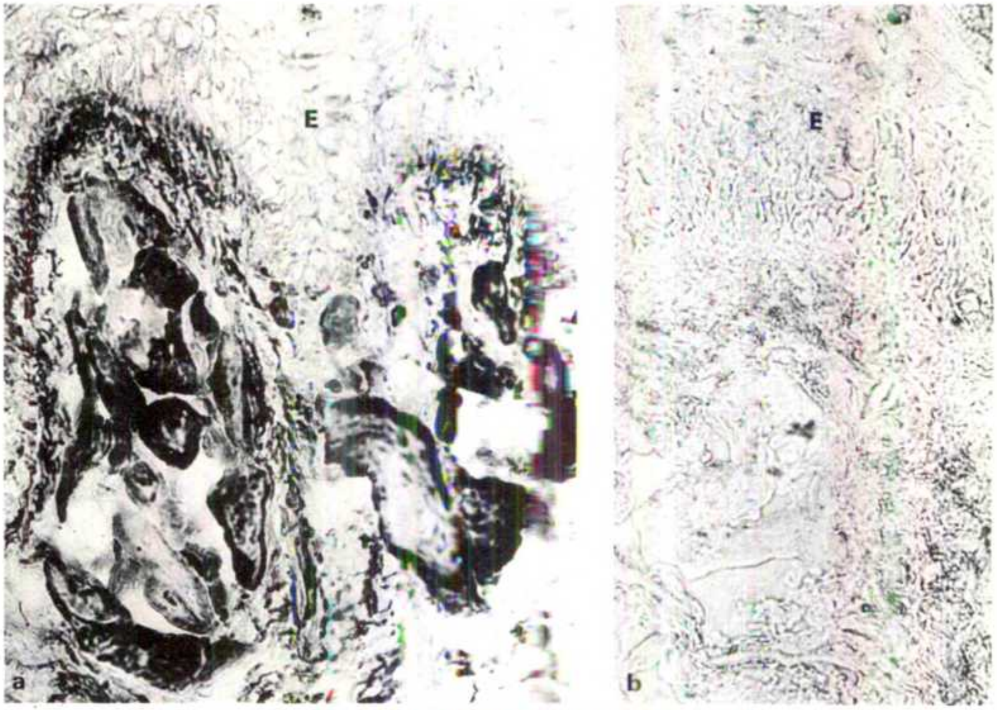

FIGURE 4. Immunoperoxidase staining of EPP with HRP-anti-SAP. Specific staining of thickened vascular walls and oxytalan fibres in the papillary dermis (a) is inhibited by preabsorption of the HRP-anti-SAP with isolated pure SAP (b). Staining is most intense at the peripheral (abluminal) borders of the vascular structures. E = epidermis. (× 313).

convoluted, greatly thickened blood vessel walls in the papillary and upper reticular dermis (Figs 1–3), which was inhibited completely by preabsorption of the fluors with pure SAP or fibronectin. There was in addition specific staining of dermal elastic tissue fibres with anti-SAP, and linear dermo-epidermal junction zone and reticular upper dermal fluorescence with anti-fibronectin, as in normal skin. An appearance of linear dermo-epidermal basement membrane zone (BMZ) fluorescence with anti-SAP was a constant finding in EPP, and was the result of confluent staining of a ramifying plexus of fine oxytalan fibres in this region (Fig. 2). In places the linear band of fluorescence seemed to separate into two distinct layers, interconnected by a network of fine fibres. A similar pattern of staining with anti-SAP in EPP was observed in immunoperoxidase studies; specific staining was again inhibited by preabsorption of the reagent with pure SAP (Fig. 4). Although staining of the abnormal vascular structures was sometimes homogeneous, the density of staining was often most intense at their peripheral (abluminal) borders, the central areas remaining relatively clear of staining.

Electron microscopy

Ultrastructure examination of skin from patients with EPP revealed the presence of many abnormal structures in the papillary and upper reticular dermis (Fig. 5). These were usually recognizable as being of vascular origin, despite the necessarily incomplete tissue fixation achieved in order to preserve the antigenicity of AP; whilst the cytoplasm of endothelial cells and pericytes was severely disrupted or absent, the nuclei of these cells were usually intact. Endothelial cell remnants were surrounded by an area of multiple concentric reduplication of the leaflets of the basal lamina. Peripheral to the basal laminar area was a zone of amorphous matrix containing microfibrillar structures and scattered randomly-orientated collagen fibres. Ultrastructural immunoperoxidase staining of EPP with anti-SAP confirmed specific binding of the peroxidase-conjugated antiserum to the walls of upper dermal blood vessels (Fig. 6).

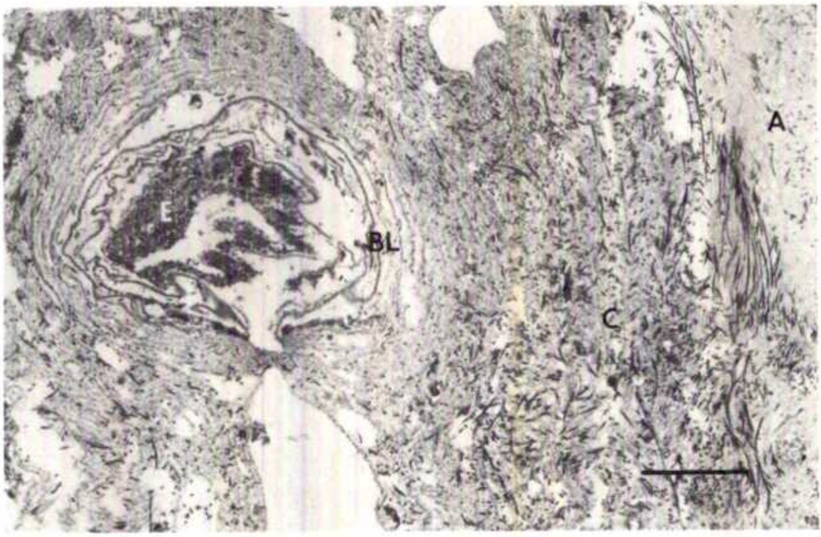

FIGURE 5. Ultrastructural appearance of papillary blood vessel cut in transverse section: note absence of specific staining in this inhibition control in which the HRP-anti-SAP was preabsorbed with isolated pure SAP. A zone of concentric reduplication of the basal lamina (BL) surrounding an endothelial cell (E) merges peripherally with an area of collagen fibres (C) and microfibrils embedded in an amorphous matrix (A). Bar = 5 μm. (× 2800).

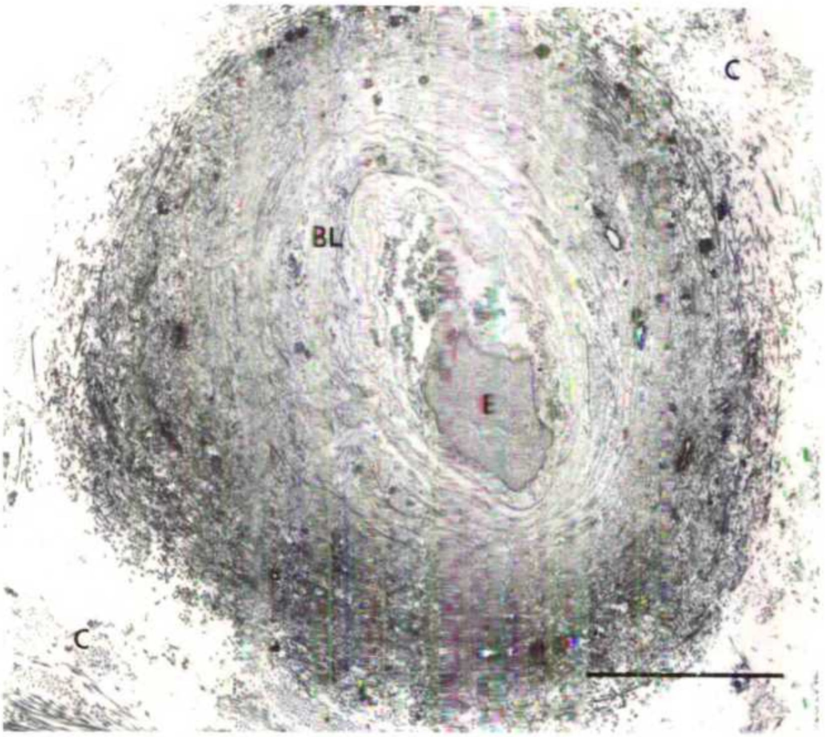

FIGURE 6. Immunoperoxidase staining of EPP with HRP-anti-SAP. Transverse section of a papillary blood vessel showing concentric reduplication of the basal lamina (BL) surrounding an endothelial cell (E). Specific staining is confined to the periphery of the vessel wall. Leaflets of the basal lamina and adjacent collagen fibres (C) are not stained. Bar = 5 μm (× 5250).

Staining was confined to the peripheral (abluminal) areas of the blood vessel walls corresponding to the region of greatest concentration of the amorphous matrix containing microfibrils and collagen fibres. Leaflets of the reduplicated basal lamina and collagen fibres outside the confines of the vessel walls were not stained. Specific staining with anti-SAP was observed in a linear pattern around certain blood vessels in the deep reticular dermis at the site of the external elastic lamina, and was also noted in association with elastic fibre microfibrils throughout the dermis as in normal skin.

DISCUSSION

Sunlight or long wave ultraviolet light (UVA) irradiation of the skin of patients with EPP (Gschnait *et al.*, 1975) and of mice with griseofulvin-induced protoporphyria (Konrad *et al.*, 1975) results in extensive damage to dermal vascular endothelial cells, leakage of intravascular contents, and accumulation of amorphous foamy material in the subendothelial area. The exact mechanism of the vascular damage is uncertain although interactions between protoporphyrins, UVA, lysosomes (Allison, Magnus & Young, 1966) and/or complement components (Lim & Gigli, 1981) have been postulated. Repeated UVA exposure is followed by concentric reduplication of the vascular basal lamina and the appearance of a finely fibrillar substance permeating and surrounding thickened vessel walls (Ryan & Madill, 1968; Anton-Lamprecht &

Meyer, 1970; Epstein *et al.*, 1973; Nonaka *et al.*, 1975; Gschnait *et al.*, 1975; Kint & de Weert, 1978; Hönigsmann *et al.*, 1976). The present study has shown that this characteristic thickening and reduplication of blood vessel walls in EPP involves local deposition of AP and fibronectin, in addition to that of type IV collagen and laminin as reported elsewhere (Wick *et al.*, 1979).

Immunofluorescence staining with anti-SAP of exposed skin in EPP revealed confluent fluorescence of a ramifying plexus of oxytalan fibres along the dermo-epidermal junction. Similar continuous dermo-epidermal BMZ fluorescence with anti-SAP is present in exposed lesional skin of patients with discoid lupus erythematosus (LE) (Breathnach *et al.*, 1982b) but is not found in normal skin (Breathnach *et al.*, 1981a). An increase in the number of oxytalan fibres may therefore contribute to the thickening of the PAS-positive light-microscopic basement membrane seen in EPP and LE, and which ultrastructurally is composed of the sub-basal lamina fibrous zone (Briggaman & Wheeler, 1975). A faint linear band of fluorescence above and connecting with the plexus of oxytalan fibres may have represented the termination of these fibres along the basal lamina, into which they are inserted (Kobayasi, 1968; Briggaman & Wheeler, 1975; Cotta-Pereira, Guerra Rodrigo & Bittencourt-Sampaio, 1976).

Fibronectin is a normal constituent of all vascular basement membranes (Stenman & Vaheri, 1978; Fyrand, 1979; Couchman *et al.*, 1979; Ruoslahti *et al.*, 1981) and might accordingly be expected to be found in increased quantities in reduplicated vascular basement membranes in EPP. AP, however, is not normally present in basement membranes of cutaneous blood vessels (Breathnach *et al.*, 1981b, 1982a). AP was localized in immuno-electron microscopic studies to the peripheral (abluminal) regions of thickened vascular walls in EPP, and was not detected in association with leaflets of the reduplicated basal lamina. The area of staining with anti-SAP corresponded to the region of maximum concentration of an amorphous matrix containing randomly orientated collagen fibres and microfibrillar material. Collagen fibres do not bind antibodies to SAP (Breathnach *et al.*, 1981b, 1982a). The presence of microfibrillar material permeating the walls of dermal blood vessels is a characteristic feature of the histology of EPP (Ryan & Madill, 1968; Anton-Lamprecht & Meyer, 1970; Epstein *et al.*, 1973; Nonaka *et al.*, 1975; Gschnait *et al.*, 1975; Kint & de Weert, 1978). The specific staining of thickened vascular walls with anti-SAP observed in EPP may have been associated with this microfibrillar material, as AP is specifically associated with microfibrils in normal human elastic tissue (Breathnach *et al.*, 1981b, 1982a).

At present it is not clear whether the AP and fibronectin in the abnormal vessel walls are derived from SAP and plasma fibronectin which have leaked from the intravascular compartment, or whether they have been synthesized locally by fibroblasts and/or vascular endothelial cells (Spark *et al.*, 1978; Yamada & Weston, 1974; Jaffe & Mosher, 1978) in response to repeated episodes of protoporphyrin-mediated photochemical injury.

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Immunofluorescence and histochemical studies of localized cutaneous amyloidosis

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SUMMARY

Lichen amyloidosus (LA) and macular amyloidosis (MA) are two forms of localized cutaneous amyloidosis in which the amyloid occurs as larger and smaller deposits respectively in the papillary dermis. The histogenesis of the amyloid of these conditions is unknown. By using an indirect immunofluorescence technique we showed that LA and MA do not react with antibodies against different previously characterized amyloid fibril proteins. These results indicate that the amyloid of LA and MA is different from other known types of amyloid. Protein AP, which was demonstrated in amyloid of MA and LA, is known to be present in all forms of amyloid and is of unknown significance. Antiserum against keratin did not react with the larger homogeneous amyloid bodies, but showed a weak reaction with some small deposits. Histochemical staining failed to show keratin in any of the tissues containing LA or MA.

In primary, myeloma-associated and secondary systemic amyloidosis, skin involvement is common (Brownstein & Helwig, 1970; Schilder, 1909; Symmers, 1956; Westermark, 1972). In addition, there are several different forms of localized amyloidosis of the skin, of which lichen amyloidosus (LA) and macular amyloidosis (MA) are the most common. Several authors have described both forms in the same patient, suggesting that they may be variants of one disease (Bedi & Datta, 1979; Brownstein & Hashimoto, 1972; Toribro *et al.*, 1975). LA is most often found as small greyish-brown hyperkeratotic papules on the front of the shins. Histologically,

Abbreviations: LA, lichen amyloidosus; MA, macular amyloidosis; AP, amyloid P-component [Amyloid protein nomenclature accepted at the Third International Symposium on Amyloidosis, Povoa de Varzim, Portugal, September, 1979 (Benditt *et al.*, 1980).]; AA, amyloid fibril protein A; AL, immunoglobulin light chain amyloid fibril protein; AλI, AλIV, AλVI, immunoglobulin light chain amyloid fibril proteins of the λI, λIV and λVI subgroups; AE_t, amyloid fibril protein from medullary carcinoma of the thyroid; ASc_1, amyloid fibril protein from senile cardiac amyloid; AD, amyloid fibril protein from localized cutaneous amyloid (not identified).

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there is hyperkeratosis and acanthosis, and amyloid deposits are found in the papillary dermis. MA presents with discrete brownish macules, commonly found around the neck and between the scapulae. Epidermal changes are minimal, and small amyloid deposits are present in the dermal papillae. The pathogenesis of LA and MA is still obscure. Some authors (Black, 1971; Black & Heather, 1972; Black & Wilson Jones, 1971; Hashimoto & Kabayasi, 1980; Kumakiri & Hashimoto, 1979) have postulated that epidermis plays an important role, while others (Ebner, 1968; Hashimoto & Brownstein, 1972; Hashimoto & Onn, 1971) have suggested that dermal fibroblasts are involved in the amyloid production. Melanocytes have also been implicated in the pathogenesis of LA and MA (Westermark, 1979)

We have now studied the nature of the amyloid in LA and MA by indirect immunofluorescence, using antibodies to previously characterized amyloid fibril proteins. Since it has been suggested that the amyloid of LA and MA is partially derived from keratin (Kumakiri & Hashimoto, 1979; Maeda *et al.*, 1982; Masu, Hosokawa & Seiji, 1981), we have also performed histochemical and immunochemical studies for keratin. Finally, the presence of different immunoglobulins, C3 and amyloid P-component (protein AP) has been demonstrated.

METHODS

We investigated skin specimens from eight patients with LA, three with MA and one with clinical and histological findings suggesting both LA and MA (Table 1). Several punch biopsies of affected skin were performed on each patient under local anaesthesia. Some tissues were washed overnight in cold 0·15 M phosphate-buffered saline (PBS), pH 7·2, before being embedded. The biopsy specimens were then either snap-frozen or embedded in paraffin by the method of Sainte-Marie (1962). After deparaffination, 5–7 μm sections were fixed in methanol–acetone (1:1), rinsed in PBS and incubated in a moist chamber with the different antisera in dilutions of 1:16 and 1:32. Normal rabbit serum was used as a control. The sections were then washed thoroughly with PBS and covered with fluorescein-conjugated (FITC) swine anti-rabbit antiserum (1:32). The slides were again washed and mounted in PBS-glycerol under

TABLE 1. Clinical data on patients with lichen amyloidosus (LA) and macular amyloidosis (MA)

Patients	Type of amyloidosis	Sex	Age	Localization	Country of origin
1	LA	Male	66	Shins	Sweden
2	LA	Female	48	Shins, upper arms	Sweden
3	LA	Female	33	Shins	Sweden
4	MA + LA	Female	23	Neck, chest, abdomen (MA), Pubic region (LA)	Sweden
5	MA	Female	67	Neck, between shoulder blades	Sweden
6	MA	Female	63	Between shoulder blades	Sweden
7	MA	Male	62	Between shoulder blades	Sweden
8	LA	Male	41	Shins	USA
9	LA	Female	71	Arms, between shoulder blades	USA
10	LA	—	—	Shins	Taiwan
11	LA	—	—	Shins	Taiwan
12	LA	—	—	Shins	Taiwan

a coverslip. All washings and incubations were carried out at room temperature for 30 min. The tissues were examined within one hour or stored in the dark at 4°C until the next day. The fluorescence Congo red method (Cornwell *et al.*, 1982) was used for some tissues.

Rabbit antibodies to IgA, IgM, IgG and C3 (DAKO Immunoglobulins, Copenhagen, Denmark) were used. Antiserum to protein AP was a gift from Dr. M. Skinner, Boston or was purchased from Atlantic Antibodies. Antiserum to human keratin from stratum corneum, purified according to Sun and Green (1979), was raised in rabbits. Anti-keratin antiserum was also kindly provided by Dr T. Sun. Previously characterized rabbit antisera to amyloid fibril protein AA, AλI, AλIV, AλVI, AE_t and ASc_1 (Cornwell *et al.*, 1977 and 1978) were also used. Keratin was also identified by the Ayoub–Shklar histochemical method (Luna, 1968).

RESULTS

In all biopsies, amyloid deposits were limited to the papillary dermis. In LA large lumps, which sometimes distended the papillae, dominated (Fig. 1). The amyloid in MA consisted of groups of small, faceted bodies intermingled with pigment-containing cells (Fig. 2). Such small bodies were also found together with larger deposits in the patient with both LA and MA and in one patient with LA (RM). The amyloid showed a typical green birefringence between crossed polars.

In all patients with LA and MA, the amyloid reacted with antisera to IgG, IgA, IgM and C3 (Fig. 3). Anti-IgM and anti-C3 antisera gave a brighter fluorescence pattern than anti-IgA and

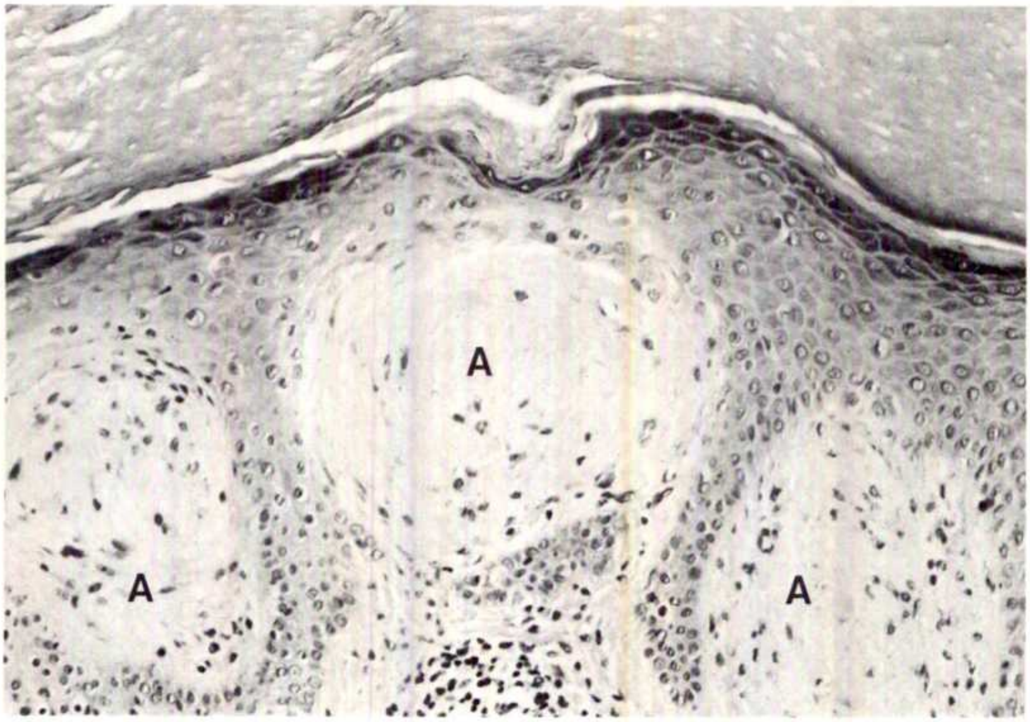

FIGURE 1. Lichen amyloidosus. The papillary dermis contains heavy deposits of amyloid (A) (Van Gieson stain, × 295).

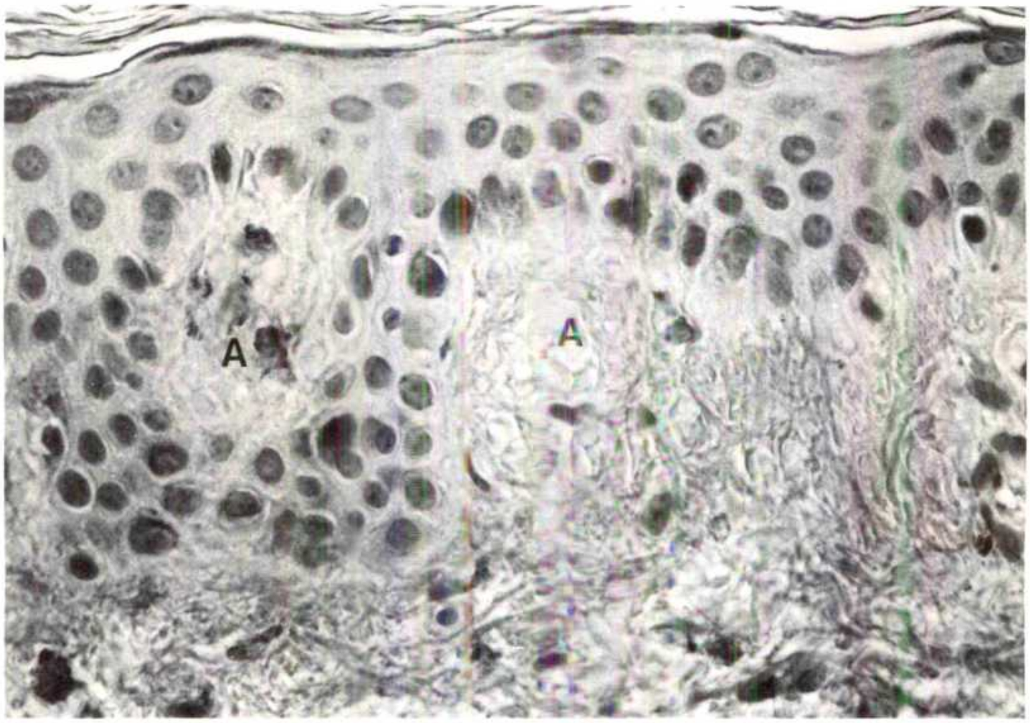

FIGURE 2. Macular amyloidosis. Small amyloid bodies (A) are seen in the papillary dermis (Van Gieson stain, × 880).

anti-IgG antisera. The fluorescence was not homogeneous but was often more intense at the periphery of the individual amyloid deposits. In one case (GS), there was a reaction of the basement membrane zone with the anti-IgM antiserum.

The reaction of anti-protein AP with amyloid of LA and MA universally resulted in a brighter and more homogeneous fluorescence pattern than that observed with the antisera to the immunoglobulins. In the dermis, elastin fluoresced intensively. There was no reaction of the amyloid with antisera to amyloid fibril proteins of the AA, AL, AE, or ASc_1 type.

The anti-keratin antiserum resulted in a bright fluorescence of all portions of the epidermis except the stratum corneum, which reacted weakly or strongly in small areas. The antiserum also reacted with the different adnexae. This pattern was found in both normal healthy controls and in patients with LA and MA. The larger homogeneous amyloid deposits of LA and most of the small amyloid bodies of MA did not fluoresce at all (Figs 4 and 5), but several of the smaller deposits in both LA and MA showed weak peripheral fluorescence (Fig. 6). There was no difference whether paraffin sections or frozen sections were used, and both anti-keratin antisera gave identical results. All controls were negative in the dermis. The weak amyloid fluorescence was seen most readily in tissues which were not washed in PBS. The unwashed tissues, however, demonstrated a similar fluorescence pattern when stained with normal rabbit serum.

Histochemical staining of washed and unwashed specimens of tissue containing LA or MA failed to show evidence of keratin in amyloid deposits although stratum corneum and epidermis reacted positively.

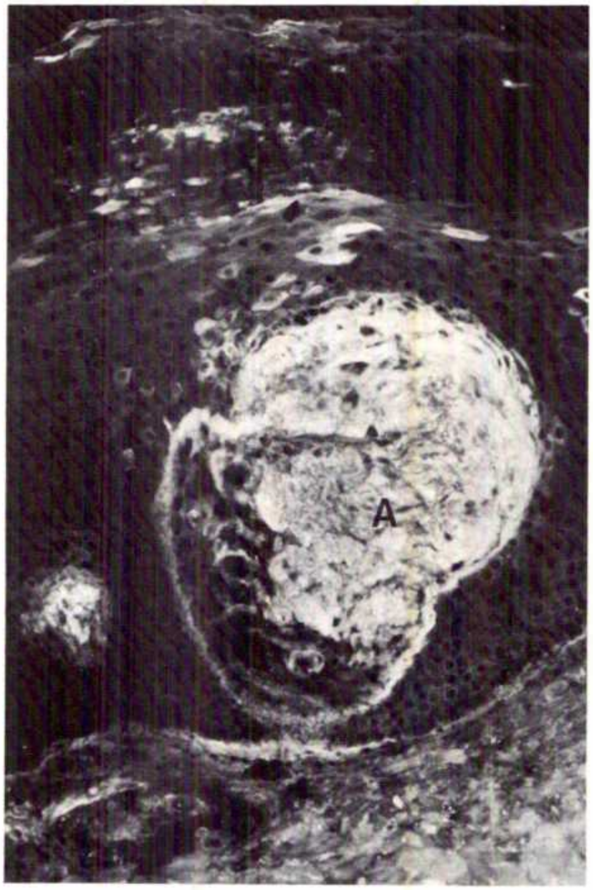

FIGURE 3. Lichen amyloidosus. Immunofluorescence reaction of anti IgG with amyloid deposits (A) (× 647).

DISCUSSION

Amyloid substance is composed predominantly of fibrils which are polymers of proteins in a β-pleated sheet conformation (Eanes & Glenner, 1968). Four different groups of amyloid fibril proteins have been characterized to date by amino acid sequence analysis: protein AA in systemic secondary amyloidosis (Benditt *et al.*, 1971); homogeneous immunoglobulin light chains or fragments thereof (protein AL) in primary and myeloma-associated systemic amyloidosis (Glenner *et al.*, 1971); prealbumin-related protein ASc_I in 'senile' cardiac amyloidosis (Sletten, Westermark & Natvig, 1980); and hormone-derived proteins (including AE_t) in amyloid of endocrine organs (Sletten, Westermark & Natvig, 1976). In the present study, we tested antisera to the amyloid fibril proteins AA, AL, AE_t and ASc_I without any reaction. These results suggest that the amyloid of LA and MA is different from any type of amyloid heretofore described and may contain a distinctive protein, tentatively designated AD (Benditt *et al.*, 1980).

The nature of protein AD is unknown. It has been proposed that the amyloid in LA and MA is derived from keratin (Kumakiri & Hashimoto, 1979). Masu *et al.* (1981) reported that an

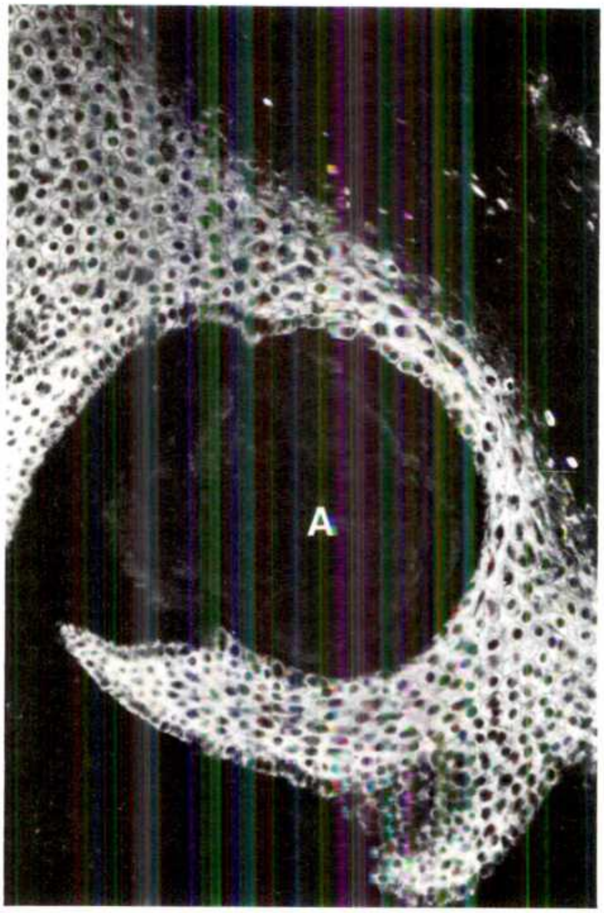

FIGURE 4. Lichen amyloidosus. Immunofluorescence micrograph demonstrating no reaction of amyloid (A) with anti-keratin (× 676).

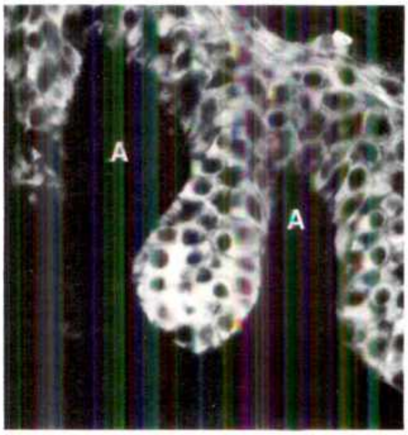

FIGURE 5. Macular amyloidosis. Immunofluorescence micrograph demonstrating no reaction of these amyloid bodies with anti-keratin (× 525).

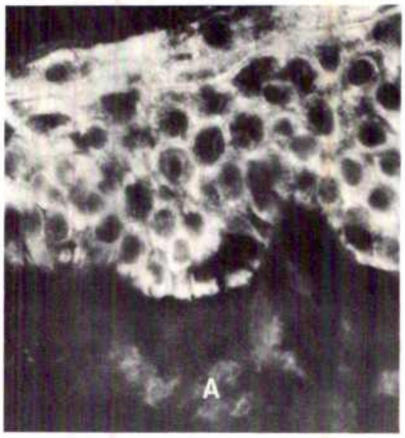

FIGURE 6. Macular amyloidosis. A possible weak reaction of amyloid with anti-keratin is shown in this immunofluorescence micrograph (× 875).

anti-keratin antiserum reacted with the amyloid in LA. In contrast, we found that the larger deposits of amyloid in LA were completely negative in immunofluorescence with this antiserum, while some small amyloid deposits, resembling Civatte bodies were slightly positive. Additionally, histochemical stains for LA and MA failed to demonstrate amyloid-associated keratin irrespective of the method of preparing, fixing or embedding the tissues. These discrepancies are difficult to interpret, and the role of keratin in the structure or formation of amyloid may require the extraction of sufficient amyloid for direct biochemical studies.

Protein AP is a normal plasma protein of the α-globulin type, the function of which is unknown (Pepys *et al.*, 1980). It has previously been found in all types of human amyloid studied and the localized amyloid of the skin does not appear to be an exception (Breathnach *et al.*, 1981b; Westermark *et al.*, 1981). Whether protein AP is absorbed in amyloid non-specifically or plays a role in the formation of amyloid fibrils is unknown (Pepys *et al.*, 1980). The reaction of antiprotein AP antiserum with elastin noted here and previously (Breathnach *et al.*, 1981b, Westermark *et al.*, 1981) depends on an immunospecific binding of the antibodies to the peripheral microfibrillar mantle of elastic fibres in the skin (Breathnach *et al.*, 1981a).

Previous studies have revealed the presence of immunoglobulins and C3 in the amyloid of LA and MA (Haberman & Montenegro, 1980; MacDonald, Black & Ramnarain, 1977; MacDonald, Fergin & Black, 1980) as well as in other amyloid substances (Husby & Natvig, 1972; Renu *et al.*, 1980). These immunoglobulins are, however, not thought to constitute an integral part of the amyloid fibril, but are probably associated with it by non-specific absorption. This conclusion is supported by the findings of MacDonald *et al.* who reported that the immunoglobulins could be removed by washing without changing the histochemical reaction of the amyloid (MacDonald *et al.*, 1980).

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Direct immunofluorescence in pyoderma gangrenosum

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SUMMARY

Direct immunofluorescence was done in fifty-one cases of pyoderma gangrenosum. Biopsy specimens were taken from the peripheral erythematous zone of the lesion. In thirty-one cases (61%), there was positive immunofluorescence, with perivascular deposition of immune reactants being the most frequent pattern (twenty-seven cases). These findings support a vasculitic pathogenesis of pyoderma gangrenosum.

Pyoderma gangrenosum was described as a clinical entity more than 50 years ago (Brunsting, Goeckerman & O'Leary, 1930), but its cause remains obscure. It is often associated with other diseases, especially bowel disorders (Perry, 1969; Greenstein, Janowitz & Sachar, 1976), arthritis (Stolman *et al.*, 1975; Holt *et al.*, 1980), abnormalities of the serum proteins (Jablonska, Stachow & Dabrowska, 1967; Powell *et al.*, 1983), blood dyscrasias (Perry & Winkelmann, 1972; Callen, Dubin & Gehrke, 1977), and chronic active hepatitis (Byrne, Hewitt & Summerly, 1976).

Several studies have shown disordered immune responses in patients with pyoderma gangrenosum (Delescluse, de Bast & Achten, 1972; Lazarus *et al.*, 1972; Miller & Dooley, 1973; Brandt *et al.*, 1977; Clayton, 1977), mainly defective cell-mediated immunity but also impaired phagocytosis by neutrophils. Histopathological examination may show a non-specific pattern of abscess formation, but evidence of vasculitis may be seen (Bishopric & Bracken, 1964; Samitz, Dana & Rosenberg, 1970; Thompson *et al.*, 1973; Dantzig, 1975; Stolman *et al.*, 1975), especially if the biopsy specimen is taken from the advancing erythematous border, where endothelial swelling of the vessels with fibrinoid necrosis and perivascular lymphocytic infiltrate is seen in more than 80% of cases (Schroeter & Su, 1980). Immunofluorescence studies of biopsied material have been mainly in the form of case reports. Often the exact biopsy site in relation to the lesion of pyoderma gangrenosum was not specified, and the results have been negative in most cases.

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We examined fifty-one patients with pyoderma gangrenosum, with biopsy specimens taken from a specific site, to determine whether there was any consistent pattern of immunofluorescence in this disease.

METHODS

Direct immunofluorescence was done in fifty-one cases of pyoderma gangrenosum. In each case, pyoderma gangrenosum was diagnosed on the basis of clinical findings of a chronic ulcerated area with a mucopurulent base and a violaceous undermined edge surrounded by a zone of erythema. Multiple biopsy specimens were taken for histopathological examination and cultures for fungus, tuberculosis, and atypical mycobacterium were performed. A single biopsy specimen for direct immunofluorescence was obtained in thirty-five cases and multiple biopsy specimens in sixteen. The biopsy technique used for direct immunofluorescence study involved a 4-mm punch biopsy of the peripheral erythematous zone surrounding the ulcer. Biopsy specimens taken from the lower leg of thirty healthy volunteers served as controls. Tissue was frozen immediately in liquid nitrogen, and within 48 h, frozen sections were stained for immunoreactants with fluorescein-conjugated goat antibody to human IgG, IgM, IgA, C3, and fibrin by a previously described technique (Beutner, Chorzelski & Jordon, 1970). Conjugates were obtained from Behring Laboratories (anti-IgG, anti-IgM, anti-IgA) and Hyland Laboratories (anti-C3 and anti-fibrin). Specificity was tested with immunoelectrophoresis and the Ouchterlony procedure. Specimens were then examined using a UVR microscope (Ortholux II), with irradiance between 320 and 490 nm and a barrier filter at 430 nm. With water-immersion lenses, slides were scanned at ×313, and the findings were confirmed at ×625.

RESULTS

Thirty-one patients (61%) had positive direct immunofluorescence, and those with multiple biopsy specimens had a higher positive yield (thirteen of sixteen, 81%) than those with a single

TABLE 1. Results of direct immunofluorescent study in fifty-one cases of pyoderma gangrenosum

No. of patients	Pattern of immunofluorescence	Immune reactants*
27	Perivascular	IgM (17), IgA (3), IgG (1), C3 (19), fibrin (16)
10	Perivascular and basement membrane zone	IgM (9), C3 (5), fibrin (5)
4	Basement membrane zone only	IgM (1), fibrin (1), C3 (2)
11	Cytoids	IgM (11), IgA (1), C3 (1), fibrin (1)
20	Negative	—

* Number of patients in parentheses.

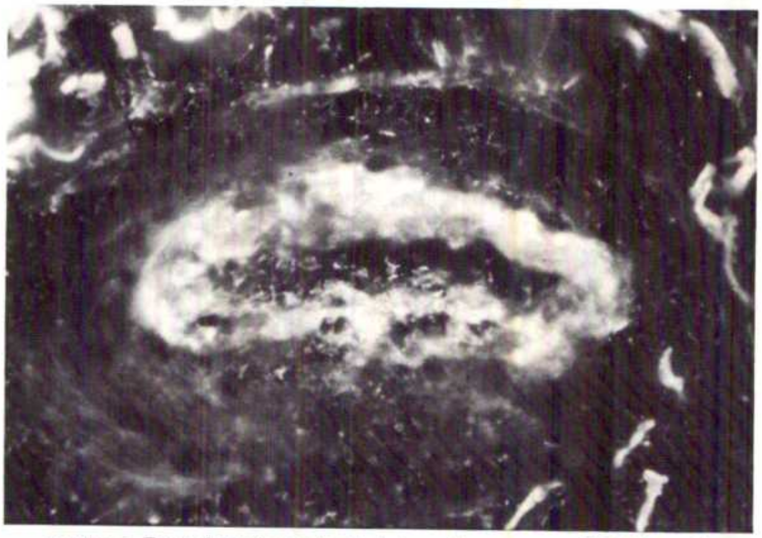

FIGURE 1. Deep dermal vessel with intense fluorescence of IgM in wall.

biopsy specimen (twenty of thirty-five, 57%). Perivascular or intravascular deposition of immune reactants was the most frequent pattern, present in twenty-seven of the thirty-one cases with positive immunofluorescence (Table 1), and IgM and C3 were the reactants most frequently seen (Figs 1 and 2). Superficial and deep vessels were involved in equal numbers, and the intensity of fluorescence varied from +1 to +3, with an average intensity of +2 (range 0 to +3). Arteriolar fluorescent involvement was found in many cases (Fig. 2). Basement membrane zone fluorescence was seen in only four cases. Fluorescence of cytoids was noted in eleven cases. Forty-one patients had another disease associated with the pyoderma gangrenosum. The presence or type of associated disease did not seem to influence the immunofluorescent findings

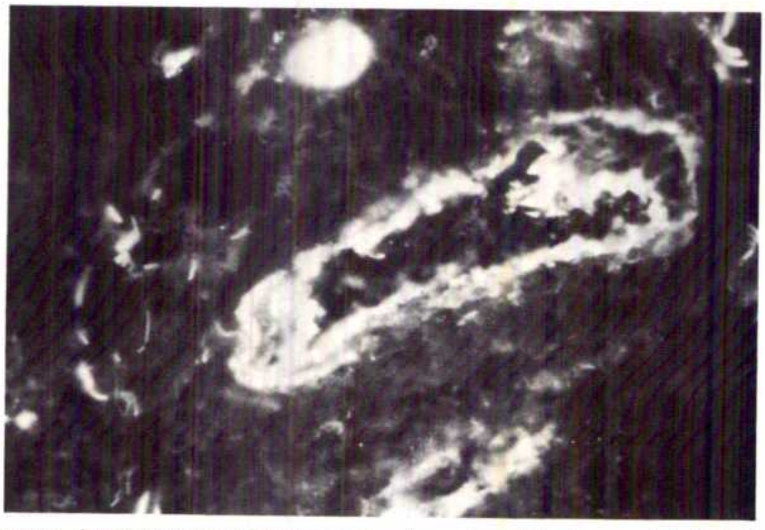

FIGURE 2. Arteriole with brightly fluorescing deposition of C3 about endothelial cells.

TABLE 2. Associated diseases in fifty-one cases of pyoderma gangrenosum in relation to direct immunofluorescent findings

Disease	Direct immunofluorescence Positive	Negative	Total
Seronegative arthritis	3	3	6
Rheumatoid arthritis	3	2	5
Crohn's disease	5	1	6
Ulcerative colitis	[illegible]	5	6
Diverticulitis	2	1	3
Monoclonal gammopathy	2	3	5
Others	8	2	10
None	5	5	10

(Table 2). In only one of the thirty control cases was there vascular fluorescence (C3). In all others, direct immunofluorescence was negative.

DISCUSSION

Our results show that when perilesional erythematous skin is biopsied, most specimens of pyoderma gangrenosum show positive direct immunofluorescence. Previous studies of direct immunofluorescence in cases of pyoderma gangrenosum have generally been negative. We found eighteen reports in the literature which described direct immunofluorescence in a total of twenty-eight cases. These were mainly case reports, and often the biopsy site was not specified or the specimen was taken from lesional skin. Lynch & Bergfeld (1978) did direct immunofluorescence in four cases of pyoderma gangrenosum and found that all were negative for IgG, IgM, IgA, complement, and fibrinogen, but they did not describe the biopsy site. 'Early lesional skin' was examined in four patients by Holt *et al.* (1980) and in two patients by Dantzig (1975); all had negative results. Delescluse *et al.* (1972) biopsied 'unaffected skin near the lesion' on two occasions in a patient with pyoderma gangrenosum, again without finding any immunoreactants, while Clayton (1977) biopsied 'perilesional skin' and had negative results. Stolman *et al.* (1975) examined material from the 'violet margin' in one patient and an unspecified site in another, again without positive results. Wustrack & Zarem (1978), Norris *et al.* (1978) and Romano & Safai (1979) all had negative findings, each in a single case of pyoderma gangrenosum, while Johnson & Lazarus (1982) found non-specific background fluorescence and 'non-specific changes of chronic inflammation' on immunofluorescent microscopic examination in two cases of pyoderma gangrenosum. Thompson *et al.* (1973) found granular deposition of IgG, β1c, and fibrin in the walls of vessels, although their patient was somewhat atypical. Texier *et al.* (1976) found intravascular deposition of IgM and C3 in perilesional skin of a patient with pyoderma gangrenosum and Crohn's disease, while Claudy, Gogue & Volle (1978) found C3 deposition in the superficial and deep vessels of lesional skin in a patient with seronegative arthritis and pyoderma gangrenosum. Reboul *et al.* (1981) showed C3 and fibrin in vessels and at the dermal-epidermal junction in a patient with associated myeloma, and Thomsen & Rothenborg (1979) found granular deposition of C3 at the basement membrane zone in a patient successfully treated with clofazimine.

Holt (1979) pointed out that deposition of immunoprotein may occur but that it may be evanescent, requiring multiple early biopsy specimens for detection. Hickman & Lazarus (1979), in their review of the aetiology and treatment of pyoderma gangrenosum, mentioned that immunofluorescence studies of skin injected with histamine were negative in two cases and stated that immunofluorescence studies of skin peripheral to the advancing border of the disease had not been performed. Clearly, there was a need to study a large number of patients with this disease, with biopsy specimens taken from a specified site to determine if any specific pattern of direct immunofluorescence can be found. Because immune reactants are rapidly destroyed in areas of intense inflammation and necrosis (Cream, Bryceson & Ryder, 1971), we biopsied the advancing erythematous peripheral border in our study.

The predominant pattern of deposition of brightly fluorescing immune reactants in and about both the superficial and deep dermal vessels in our patients is striking, as is the preponderance of IgM and C3. Fluorescence at the basement membrane zone was less intense and probably non-specific and reflects diseased vessel activity only. Taking multiple biopsy specimens increased the likelihood of positive findings; probably more than 60% of patients with pyoderma gangrenosum will have positive direct immunofluorescence if multiple biopsy specimens are taken in patients with initially negative results.

Because of recent reports suggesting that vascular fluorescence may be found in a significant number of normal persons (Baart de la Faille-Kuyper, van der Meer & Baart de la Faille, 1974; Nieboer, 1981), we used thirty healthy volunteers to act as controls for our study. The lack of positive findings in these subjects (except for one case with C3 in the superficial vessels) confirms our previous experience with normal controls (Schroeter, Conn & Jordon, 1976) and that of others (Beutner *et al.*, 1975; Blenkinsopp, Clayton & Haffenden, 1978). The significance of vascular fluorescence about areas of inflammation and ulceration is less clear, however. In patients with an underlying cause of vasculitis (for example, rheumatoid arthritis), there will be deposition of immune reactants both in vessels of the normal skin and about areas of ulceration (Conn, Schroeter & McDuffie, 1976). Dahl (1981) stated that immunoglobulins and complement are usually deposited in blood vessel walls, around areas of ulceration of any cause, and Miller & Griffiths (1982) have shown that vascular deposition of complement can be induced in normal skin following injury. However, only one of their patients had deposition of immunoglobulin in a papillary vessel in a weak to moderate reaction. Alternatively, Morel, Crickx & Civatte (1980) stated that vascular fluorescence is rare except in diseases in which immune complexes are involved in the pathogenesis. We believe that vascular fluorescence occasionally may occur as an epiphenomenon of traumatized skin or non-specific ulceration, with immune reactants acting as universal antibodies directed against antigenetically altered cells. However, in the cases that we have seen the fluorescence has been faint and the pattern inconsistent. When vessels were involved, they were almost exclusively superficial vessels. In patients with pyoderma gangrenosum, the consistent pattern of intense vascular fluorescence involving both superficial and deep vessels, including arterioles, with predominantly IgM and C3 deposition, suggests immune-mediated damage of these vessels. When considered with the lymphocytic vasculitis in these patients previously described by Schroeter & Su (1980), the presence of immune reactants in and around the vessels suggests that vascular damage may be significant in the pathogenesis of pyoderma gangrenosum.

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Interferon in suction blister fluid from psoriatic lesions

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SUMMARY

Interferon (IFN) (at least 16 units/ml) was demonstrated in suction blister fluid obtained from lesional skin in nine of thirteen patients with psoriasis vulgaris and in two patients with allergic contact dermatitis, but not in blister fluid from unaffected skin. IFN was detected in only three of the sera from the patients with psoriasis. The difference in results between the blister fluid and the sera from these patients was statistically significant ($P < 0{\cdot}025$). Evidence was obtained which indicated that the activity was probably IFN-γ (immune IFN), but the additional presence of IFN-α in some of the fluids could not be excluded. The data indicate that IFN is produced locally in the psoriatic lesions, most likely by activated T lymphocytes.

Activation of T lymphocytes by mitogens (Wheelock, 1965), antigens (Green, Cooperband & Kibrick, 1970), immune complexes (Fujibayashi, Hooks & Notkins, 1975) and antibodies against membrane components (Wussow *et al.*, 1981) is accompanied by the production of type II or immune interferon (IFN-γ), which differs from the classical type I interferons produced as a result of viral infection, IFN-α (leucocyte IFN) and IFN-β (fibroblast IFN). Recently, we have demonstrated that most T lymphocytes in psoriatic skin lesions possess Ia-like antigens, indicating that they are activated cells (Bjerke & Matre, 1982). The demonstration of IFN-γ produced locally in psoriatic lesions would support our assumption that the T lymphocytes in these skin lesions are activated cells. In this study, we have investigated the presence of IFN in suction blister fluid obtained from lesional skin and in sera of patients with psoriasis.

METHODS

Patients

Thirteen patients with psoriasis vulgaris were studied. Local treatment was interrupted for at least 3 days prior to investigation. One of the patients (T.S.) received systemic methotrexate

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therapy. We also studied two patients with contact allergy, reacting strongly to patch tests with bichromate and paraphenylenediamine, respectively. Four healthy individuals served as controls.

Collection of blister fluids and sera

Suction blisters were produced using the Dermovac suction blister device purchased from Instrumentarium, Helsinki, Finland (Kiistala, 1968). The pressure within the cup was maintained at 175 mm Hg below atmospheric pressure. When suction blisters were generated in psoriatic lesions, considerable leakage often occurred through the fragile blister roofs into the cup. This fluid proved to be sterile, and was pooled with the fluid obtained within the blisters. In the two patients with contact allergy, a skin area on the upper part of abdomen, approximately 25 cm^2, was sensitized with the allergen for 22 h. Suction blisters were produced at this test site 72 h after application of the allergen. From each patient, from 0·5 to 1·5 ml of blister fluid was obtained.

Similarly, suction blisters were generated in normal appearing skin at least 10 cm away from the lesions, and in the skin of normal controls. Serum samples were obtained from all patients at the time blister fluids were collected.

All samples of blister fluid and serum were stored at −70°C until use.

Interferon assay

Interferon activity was tested by an infectivity inhibition micromethod (Dahl & Degré, 1972), employing human embryonic lung fibroblast cells in their fifth to fifteenth passage, and vesicular stomatitis virus as the challenge virus. The antiviral activity was calculated as the reciprocal of the highest dilution preventing development of cytopathogenic effect in 50% of the cells at the time when control cells were completely destroyed. One unit of the international standard preparation G-023-901-527 was equivalent to 1–1·3 units in our assay system. Based on previous experience from our laboratory (Degré, Dahl & Vandvik, 1976) and on that of others (Hooks *et al.*, 1979), only sera containing antiviral activity of 16 units/ml or higher were considered positive. The same definition of positivity was used for the blister fluids.

To characterize the antiviral activity, representative positive samples were tested for activity on heterologous cells and for trypsin and heat sensitivity. Samples were also adjusted to pH 2 at 4°C for 2 days, readjusted to pH 7·2 and then tested in the same system. Treatment at pH 2 eliminated the antiviral activity of a human IFN-γ preparation received from Dr Marc De Ley, University of Louvain, Belgium, but it did not affect the activity of a human IFN-α preparation given to us by Dr Kari Cantell, National Institute of Public Health, Helsinki, Finland.

Specific anti-human IFN globulin, prepared against human IFN-α (rabbit No. 24) was obtained from the Antiviral Programme of National Institute of Allergy and Infectious Diseases, Bethesda, MD., U.S.A. A dilution of 1 in 10,000 of this preparation neutralized 10 units of human IFN-α. Neither anti-β nor anti-γ IFN was available when this study was performed.

Statistics

Statistical evaluation was performed using the Wilcoxon two-sample test.

RESULTS

IFN (at least 16 units/ml) was detected in blister fluids of lesional skin from nine of thirteen of

TABLE I. Interferon in blister fluids and sera from patients with psoriasis vulgaris and contact dermatitis

			Interferon (units/ml)		
			Blister fluid		
Patients	Age	Sex	Lesional skin	Unaffected skin	Serum
Psoriasis vulgaris					
T.S.	32	M	< 16	n.t.*	< 16
E.S.	60	M	35	n.t.	< 16
H.J.	67	M	28	< 16	< 16
S.T.	37	M	45	n.t.	< 16
I.B.	31	M	< 16	< 16	< 16
K.M.	48	M	18	< 16	< 16
S.H.	72	M	100	< 16	< 16
M.O.	59	M	22	< 16	< 16
K.L.	32	F	< 16	< 16	22
O.K.	39	M	< 16	< 16	28
H.B.	70	M	71	n.t.	28
M.M.	29	M	35	n.t.	< 16
J.B.	27	M	56	n.t.	< 16
Contact dermatitis					
S.M.	65	M	18	< 16	22
O.E.	59	M	56	< 16	< 16
Normal controls					
n = 4				< 16	n.t.

* n.t. = not tested

the patients (69%) with psoriasis vulgaris and from both patients with allergic contact dermatitis (Table I). Blister fluid of lesional skin from the patient receiving methotrexate therapy (T.S.) did not have antiviral activity. IFN was not detected in any of the blister fluids from unaffected skin or from the skin of normal controls. IFN was detected in the sera of three out of thirteen patients (23%) with psoriasis vulgaris and in serum from one patient with contact dermatitis. The difference between the number of positive blister fluids obtained from lesional skin and the corresponding sera is statistically significant ($P < 0{\cdot}025$).

The antiviral activity was characterized as IFN by its species specificity (since no activity was obtained when tested in mouse L-929 cells), by its trypsin sensitivity and by its lack of direct neutralization of the virus.

The antiviral activity in four positive blister fluids tested was strongly reduced by pH 2 treatment and was resistant to treatment at 56°C for 30 min, indicating that the activity can be defined as IFN-γ. However, since anti-IFN-α strongly reduced the activity in one of three blister fluids, there might also be an admixture of IFN-α in some.

DISCUSSION

The results showed that most suction blister fluids from lesional skin of psoriasis contained antiviral activity characteristic of IFN, whereas antiviral activity could not be demonstrated in blister fluids from unaffected skin. To our knowledge, IFN has not been assayed previously in blister fluid from either normal or lesional skin. For serum, titres ≥ 16 units/ml were considered to be significant for the presence of IFN in blister fluid. By this criterion, none of the blister fluids from unaffected skin contained IFN. Only one of the psoriatic patients (H.B.) with IFN in blister fluid from lesional skin, also had IFN in the serum. The data obtained therefore indicate that IFN is produced locally in the psoriatic lesions. There might also have been local IFN production in the two patients with allergic contact dermatitis.

The results of characterization experiments indicated that the antiviral activity was mainly due to the presence of IFN-γ, but there might have been some admixture of IFN-α.

IFN-γ is apparently produced by different lymphocytic cells in cooperation with such accessory cells as monocytes and macrophages (Sonnenfeld, 1980). T lymphocytes are probably the main producers of IFN-γ (Epstein, Kreth & Herzenberg, 1974). Krammer *et al.* (1982), examining concanavalin A-activated mouse spleen cells, found that IFN-γ was mainly released from cytotoxic T lymphocytes. We have recently demonstrated both helper and suppressor/cytotoxic T lymphocytes in psoriatic lesions by the use of monoclonal antibodies (Bjerke, 1982). Since the majority of these T lymphocytes probably are activated cells (Bjerke & Matre, 1982), and since few B lymphocytes are present (Bjerke, Krogh & Matre, 1978), it seems likely that the IFN demonstrated is produced by T lymphocytes. Thus, the results give further support to the hypothesis that T lymphocytes in psoriatic lesions are actively involved in a cell-mediated immune reaction, which might be of importance in the pathogenesis.

IFN-γ has been shown to have several immunomodulatory activities, including both enhancing and suppressive effects (Sonnenfeld, 1980). The *in vivo* significance of these observations is, however, unclear.

IFN-γ production in psoriatic lesions may be the result of sensitized T lymphocytes reacting with an unknown antigen. The recent demonstration of suppressor/cytotoxic T lymphocytes in all layers of the psoriatic epidermis (Bjerke, 1982) could imply that these T lymphocytes react with antigens in stratum corneum. The possible presence also of IFN-α in some of the blister fluids is of particular interest in the light of a postulated viral aetiology in psoriasis (Hellgren *et al.*, 1980; Guilhou *et al.*, 1982). Recently, we demonstrated virus-like particles in blister fluid from a patient with psoriatic erythroderma (unpublished data).

IFN-γ has been demonstrated in the sera from patients with autoimmune disease (Hooks *et al.*, 1979). In our study, there was antiviral activity in the sera from 23% of the patients with psoriasis. However, since 16% of blood donors also had IFN-γ in serum (Degré, Mellbye & Clarke-Jensen, 1982), extended studies are necessary to determine the significance of this finding.

The present results are considered to be preliminary. The relation between clinical conditions (*e.g.* age of onset, extent of lesions, disease activity, response to treatment) and IFN in blister fluids needs to be studied further.

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Plasma zinc in psoriasis: relation to surface area involvement

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SUMMARY

Plasma zinc, serum albumin and alkaline phosphatase (a zinc-dependent enzyme) were measured in thirty-five psoriatics and their age- and sex-matched controls. No significant difference was seen between these two groups as a whole, but psoriatics with less than 10% surface involvement had significantly higher mean plasma zinc levels than their respective control group. Psoriatic patients also showed a relationship between the extent of surface involvement and the plasma zinc level, those with more extensive involvement having lower levels than those with minimal involvement.

The differences were not due to disturbances in serum albumin, and no abnormalities in serum alkaline phosphatase were observed.

In the past decade there have been several publications concerning plasma zinc levels in psoriasis. These originated from the finding in animals of psoriasiform skin disturbances attributed to systemic zinc deficiency. A psoriasiform component has since been described as part of the histopathology of the lesions of zinc deficiency in man (Montgomery, 1967).

The studies of plasma zinc in psoriatics resulted in conflicting results with lowered (Voorhees *et al.*, 1969; Greaves & Boyde, 1967), and normal (Withers *et al.*, 1968) levels recorded. A possible reason for conflicting results was the varying surface area involvement in subjects chosen (Greaves 1970) with significant loss of zinc in psoriatic scale (Ponomareva, 1966; Molin & Webster, 1973). Because of the potential relationship between zinc status and surface area involvement we decided to investigate this possibility further by assessing plasma zinc in patients in whom surface area involvement had been carefully tabulated.

Plasma zinc was used as the measure of zinc status (Prasad, 1976). Since most zinc is bound to serum albumin (Boyett & Sullivan, 1970), and this readily exchangeable fraction is believed to be important in zinc transport to tissues (Vallee, 1959), we also measured serum albumin levels.

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Plasma zinc is known to be disturbed in conditions with depressed serum albumin levels (Halsted & Smith, 1970) and hypoalbuminemia has been found in extensive exfoliative states (Shuster & Wilkinson, 1963). Serum alkaline phosphatase was also measured as disturbances in zinc metabolism might be reflected by changes in this zinc metallo-enzyme (Kirchgessner, Roth & Weigand, 1976).

METHODS

Plasma zinc was estimated in thirty-five psoriatic patients and in thirty-five age- and sex-matched healthy controls after an overnight fast. Surface area involvement was recorded by the 'rule of nines', subjects being divided into those with clearly more than 10% involvement, less than 10% involvement, and a third group with 10% involvement. Division into three categories was considered necessary as a significant proportion of our patients had approximately 10% surface area involvement making division into two groups of less and more than 10% involvement difficult.

Plasma zinc was measured at 08.00 hours in all subjects, under fasting conditions, in view of the diurnal variation of plasma zinc known to exist in normal individuals (McMillan & Rowe, 1982).

As a measure of the variability of plasma zinc in psoriatics, the measurement was repeated at day 5 after hospital admission in eleven patients and at day 7 in eight patients. Patients who had received topical medications containing zinc in the month before admission were excluded from the study, obviating the possibility of percutaneous absorption of zinc complicating any findings (Hallmans, 1978). Because of this latter possibility all initial plasma zinc levels were taken in the first 24 h of hospital admission before administration of topical zinc-containing medications. The only exception to this was the sequential measurements taken at day 5 and day 7 (mentioned above).

Individuals with conditions known to alter plasma zinc levels (Halsted & Smith, 1970) or with other dermatoses, or with any abnormality in routine liver function or renal function tests were excluded from the study.

Plasma zinc and serum albumin were measured as described elsewhere (McMillan & Rowe, 1982).

The age- and sex-matching data of the study and control group are given in Table I.

RESULTS

The assessment of variability of plasma zinc showed no significant variation (day 1–7).

TABLE I. Age and sex of subjects studied

	Age of psoriatics (years)			Age of controls (years)		
	Mean ± s.e.m.	No.	Sex	Mean ± s.e.m.	No.	Sex
Total	40·7 ± 2·7	35	22 M, 13 F	40·4 ± 2·6	35	22 M, 13 F
< 10% Involvement	42·1 ± 4·0	15	11 M, 4 F	41·5 ± 3·9	15	11 M, 4 F
10% Involvement	32·8 ± 5·6	8	5 M, 3 F	32·6 ± 5·8	8	5 M, 3 F
> 10% Involvement	44·4 ± 4·5	12	5 M, 3 F	44·0 ± 4·4	12	5 M, 3 F

The plasma zinc results in the total psoriatic group, subgroups with different surface area involvement and appropriate control groups are shown in Table 2. The total psoriatic group (mean plasma zinc 14·4 μmol/litre ± 0·4 s.e.m) did not differ significantly from the total control group (mean plasma zinc 14·0 μmol/litre ± 0·5 s.e.m). Although the mean plasma zinc levels of psoriatics with more than 10% surface area involvement (mean 13·3 μmol/litre ± 0·8 s.e.m.) and 10% surface area involvement (mean 13·8 μmol/litre ± 0·7 s.e.m.) were lower than respective control groups (mean 14·7 μmol/litre ± 1·0 s.e.m.; mean 14·8 μmol/litre ± 1·1 s.e.m.) these differences did not reach significance. The results in psoriatics with less than 10% surface area involvement (mean 15·6 μmol/litre ± 0·6 s.e.m.) differed from respective controls (mean 12·9 μmol/litre ± 0·5 s.e.m.)—$P < 0{\cdot}01$. A comparison of the subgroups of controls showed no significant differences in mean plasma zinc levels. This finding served as a control when psoriatic subgroups were compared with each other.

Psoriatics with more than 10% surface area involvement had a significantly lower plasma zinc (mean 13·3 μmol/litre ± 0·8 s.e.m.) than those with less than 10% surface area involvement (mean 15·6 μmol/litre ± 0·5 s.e.m.)—$P < 0{\cdot}05$. This relationship to surface area involvement was also apparent when the psoriatics with 10% or more surface area involvement (mean 13·5 μmol/litre ± 0·6 s.e.m.) were compared with those with less than 10% surface area involvement (mean 15·6 μmol/litre ± 0·6 s.e.m.)—$P < 0{\cdot}02$.

With the adequate age matching obtained (Table 1), an age-mediated effect could not have accounted for the difference between psoriatics and controls. In addition, the correlation between age and plasma zinc levels was calculated in controls and found not to be significant in the age range studied in males, females, and the total group. In addition no significant correlation was found between plasma zinc and age in the psoriatic group.

The results of serum albumin estimation in the various groups are shown in Table 3. No significant differences existed between total or psoriatic groups or their subgroups. Small differences in serum albumin levels might nonetheless have had an effect on the levels of plasma

TABLE 2. Plasma zinc (mean ± s.e.m.) in psoriatics and controls

Group	Psoriatics	Controls	Student's t test
Total Group	14·4 ± 0·4	14·0 ± 0·5	n.s.
< 10% Involvement	15·6 ± 0·6	12·9 ± 0·5	$0{\cdot}001 < P < 0{\cdot}01$
10% Involvement	13·8 ± 0·7	14·8 ± 1·1	n.s.
> 10% Involvement	13·3 ± 0·8	14·7 ± 1·0	n.s.

TABLE 3. Serum albumin in psoriatics and controls

Group	Psoriatics	No.	Controls	No.
Total	39·5 ± 0·8	(33)	40·0 ± 0·7	(29)
< 10% Involvement	38·4 ± 0·8	(15)	38·3 ± 1·1	(12)
10% Involvement	43·1 ± 2·0	(8)	42·0 ± 0·7	(8)
> 10% Involvement	38·5 ± 1·2	(10)	40·4 ± 1·0	(9)
⩾ 10% Involvement	40·6 ± 1·2	(18)	42·0 ± 0·7	(17)

TABLE 4. Zinc:albumin ratios of psoriatics and controls

Group	(Mean ± s.e.m.)			
	Psoriatics	No.	Controls	No.
Total	0·36 ± 0·01	(33)	0·34 ± 0·01	(30)
< 10% Involvement	0·40 ± 0·02	(15)	0·33 ± 0·01	(12)
10% Involvement	0·32 ± 0·02	(8)	0·35 ± 0·03	(8)
> 10% Involvement	0·33 ± 0·02	(10)	0·35 ± 0·03	(10)
⩾ 10% Involvement	0·33 ± 0·01	(18)	0·35 ± 0·02	(18)

zinc recorded. Zinc: albumin ratios (ZAR) were therefore calculated and these showed a similar trend to results obtained using plasma zinc alone (Table 4).

A significant positive correlation occurred between plasma zinc and serum albumin in controls ($r = 0{\cdot}4$, $P < 0{\cdot}05$), but not in psoriatics ($r = 0{\cdot}2$).

No significant difference in alkaline phosphatase levels occurred in any of the groups studied.

DISCUSSION

The most likely explanation of the depression of plasma zinc with increasing surface area involvement by psoriasis is zinc-depletion secondary to loss of zinc through exfoliation. The alternative possibility is that disturbances of zinc status might actually be causative in producing more severe degrees of surface area involvement.

Alternatively redistribution of plasma zinc might be involved. Redistribution to the liver has been shown to cause alterations in plasma zinc in animals (Kampschmidt & Upchurch, 1970) and the histological abnormalities shown in psoriatic liver biopsies (Roenigk *et al.*, 1971) suggest that hepatic disturbances might partially account for the changes observed.

The possibility that altered zinc status might contribute to the pathology of psoriasis is worth considering in view of the finding that psoriasiform lesions are associated with systemic zinc deficiency in several species of animals and in man. However, the failure of two clinical trials of zinc therapy to improve psoriasis argues against this view. The possibility of a block in zinc metabolism should also be considered. The finding of a raised mean plasma zinc in the psoriatic group with less than 10% involvement, when compared with respective controls, would certainly be compatible with a metabolic block. However, the level of plasma zinc in the respective controls was lower than other control groups making this apparent difference dubious. This possibility should be pursued, however, as raised plasma zinc levels have been recorded in psoriasis in one other study (Tsambaos & Orfanos, 1977).

The measurements of alkaline phosphatase (a zinc-dependent enzyme) failed to show any difference between any of the groups studied but the results are difficult to interpret. If the depression of plasma zinc associated with more widespread disease is due to zinc depletion rather than redistribution it may be that the degree of depletion in the group studied was not sufficient to cause an alteration in serum alkaline phosphatase. It is difficult to predict what would happen to levels of this enzyme if a block in zinc metabolism or a redistribution of zinc occurs in psoriasis. Measurements of alkaline phosphatase or other zinc-dependent enzymes should probably be performed in any future studies of zinc therapy in psoriasis.

Recent work in zinc-deficient animals has suggested that zinc may be involved in vitamin A release from liver (Saraswat & Arora, 1972). The findings of abnormalities of zinc metabolism in psoriasis, the relationships between zinc and vitamin A metabolism, and the therapeutic efficacy of vitamin A derivatives in a proportion of cases of psoriasis are of interest. They suggest that studies involving simultaneous measurement of plasma zinc, vitamin A and retinol binding protein levels in psoriasis in humans would be worth pursuing, especially in the context of a possible metabolic block.

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Irritant reactions to dithranol in normal subjects and psoriatic patients

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SUMMARY

The irritant reaction to dithranol on normal forearm skin of forty psoriatics and forty controls was investigated by skin contact thermometry and ID50 values were calculated for each skin type (I to IV). We concluded that there is no difference between the irritant effects of dithranol in psoriatics and normal subjects. There was no correlation between irritant dose threshold and minimal erythema dose (MED) to ultraviolet radiation (UVR) but skin type I patients are the most sensitive. The irritant response to dithranol can be partially blocked by prior treatment with indomethacin.

It is commonly believed that fair-skinned individuals who easily burn in sunlight (types I and II) do not tolerate the irritant effects of dithranol (anthralin) as well as do people with a darker complexion who tan easily. Misch *et al.* (1981) reported that there was no correlation between skin type and the erythema produced by pure anthralin. Kersey *et al.* (1981) reported that the uninvolved skin of psoriatics differs from the skin of normal subjects in its inflammatory response to dithranol.

In the present study we have investigated the skin irritancy caused by dithranol BP to determine: (a) its relationship to skin type and ultraviolet light sensitivity; (b) possible differences in reactivity between the uninvolved skin of psoriatics and the skin of normal individuals; and (c) whether the reaction could be modulated by indomethacin administration.

METHODS

Subjects

The inflammatory response to dithranol BP of forty non-psoriatic subjects with normal skin and forty psoriatics was studied. There were twenty subjects (ten controls and ten psoriatics) in each skin type I, II, III and IV of the Boston classification (Rogers *et al.*, 1979). Fresh solutions of

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dithranol BP in acetone were made and 25 μl were added to the paper discs of AL patch test discs discs (supplier: TROLAB Mellerup Denmark), and the test solution was used within 2 h of preparation. The concentration range tested was 0·1% to 1·0%. After evaporation of the acetone, the patch tests were applied to the inner aspects of both forearms in the controls and to the normal skin of the same sites in psoriatics. After remaining occluded for 24 h the patch tests were removed, leaving discrete brown stained areas on the forearms.

Assessment of irritancy

Irritant reactions to colourless substances are usually noticed by the erythema they produce in the skin. Because of the difficulty in detecting the presence of erythema in the stained areas, we used the increase in skin temperature at the site of dithranol application as compared with that in untested skin nearby.

Skin temperature measurements were taken with a chromium-aluminium thermocouple linked to a Comark electronic thermometer (Supplier, Comark Electronics Ltd. Littlehampton, West Sussex). The thermocouple sensor was attached to the skin with an adhesive plaster. Three readings were taken for each patch test site, one at the centre of the stained area and two others lateral to the edge of the dithranol staining. The difference between the reading at the centre of the patch test site and the average of the two peripheral control readings was the temperature rise used to assess the presence of irritation due to dithranol. The actual reading taken for each skin temperature measurement was the highest steady reading observed always reached within 10 s of application of the thermocouple probe

Response to ultraviolet radiation (UVR)

At the same time as the patch testing was performed each subject was irradiated on the normal skin of the upper back with UVR from a Hohensonne 460 W mercury vapour lamp (emission spectrum 250–380 nm) at a lamp/subject distance of 75 cm in order to determine the minimal erythema dose (MED) to mixed UVR for each subject. Irradiated sites were read at 24 h.

RESULTS

The change in skin temperature at each patch test site was recorded for each subject and a log dose–response curve plotted. Figure 1 shows such curves for three subjects, the roman numerals referring to the skin type of the subjects. An irritant reaction is caused by a concentration of the irritant which exceeds the threshold to produce a reaction. This threshold is known as the minimal irritant dose (MID) and in the testing of materials that do not stain is usually taken as the least concentration that causes an obvious confluent erythema. In our experiment we have arbitrarily defined the minimal irritant dose as the lowest concentration of dithranol to raise the skin temperature at the tested site by 0·5°C. The MID was recorded for each subject. The results were then analysed by the methods of probit analysis described by Kligman & Wooding (1967) for assessing the effects of strong irritants on human skin.

Reproducibility

Table 1 shows the reproducibility of the thermometric method used. Repeat patch testing at different sites on the forearms of ten patients produced the identical irritant threshold in seven cases and to within one test dose in the other three.

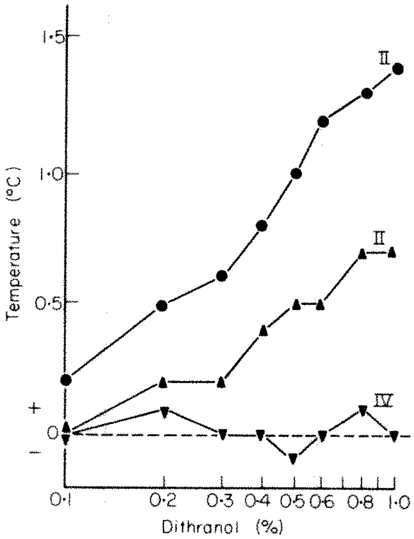

FIGURE 1. Change in skin temperature with increasing dithranol concentrations in three different subjects. II and IV refer to skin types.

MID in different skin types

Figures 2 and 3 show the percentage of responders for each concentration tested in the controls and psoriatics. Similar graphs were constructed for each skin type group. The ID50 value—(the concentration required to irritate 50% of the population tested)—and the 95% confidence limits are shown in Fig. 4. These results show that there is no difference between the threshold for irritancy between psoriatics and matched controls taken together as a group, but that skin type I control and skin type I psoriatics have the lowest threshold for irritancy which is significantly different from all other groups at the 95% confidence limit level for controls only.

TABLE 1. Comparison of irritant threshold on repeat testing in nine subjects

	MID% dithranol concentration	
Subject's skin type	Initial	Repeated
I	0·1	0·1
I (Psoriasis)	0·1	0·1
I	0·5	0·5
II	0·2	0·3
III	0·2	0·3
III (Psoriasis)	0·5	0·4
IV (Psoriasis)	0·1	0·1
IV	1·0	1·0
IV	1·0	1·0

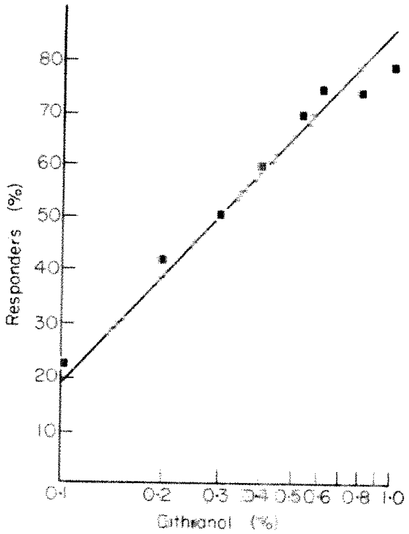

FIGURE 2. Log dose–response curve for controls ($n=32$).

Relationship of dithranol irritancy to UV-induced erythema

No significant correlation was found between irritant threshold and MED to mixed UVR in either psoriatics ($r=0{\cdot}1$) or controls ($r=0{\cdot}15$).

Effect of indomethacin administration on dithranol irritancy

Blocking the irritant reaction was attempted in five dithranol sensitive subjects with indomethacin. Five subjects with a known low threshold for irritancy were given indomethacin 25 mg eight hourly, 24 h before repeat patch testing and continued until the temperature recordings were made. Five control subjects who were not treated with indomethacin also had

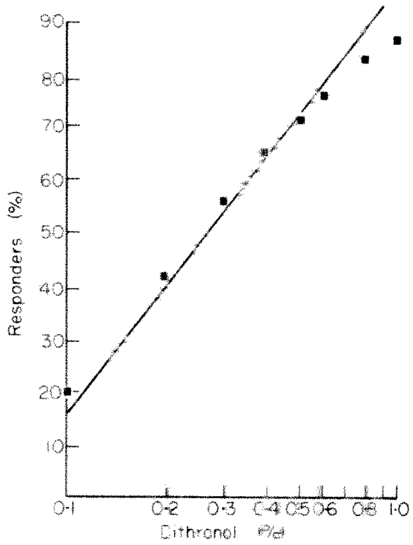

FIGURE 3. Log dose–response curve for psoriatics ($n=33$).

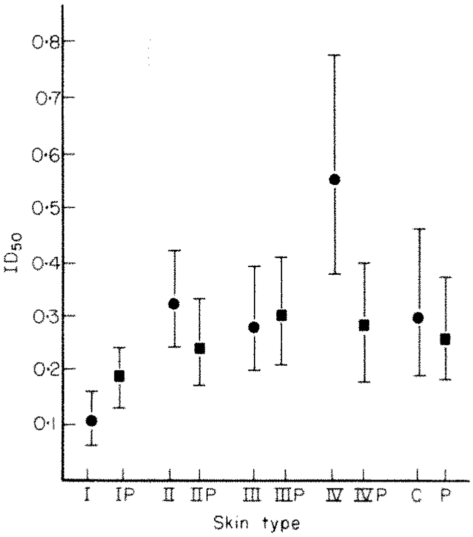

FIGURE 4. Comparison of ID50 values and skin type. (c) All controls and (p) all psoriatics (bars indicate 95% confidence limits).

repeat patch tests. Table 2 shows the results. Indomethacin blocked the irritant reaction in four patients and raised the irritant threshold in the other; no change in irritant threshold was found in the controls.

DISCUSSION

We have found skin contact thermometry an accurate and practical method for assessment of skin irritation caused by dithranol. Attaching the small thermocouple to the skin by adhesive tape enables a steady state condition to be achieved within a few seconds and eliminates problems of uneven application pressure, local convection and the need for humidity correction. In previous studies the amount or intensity of an irritant response has been measured by grading erythema on an analogue scale, measuring skin temperature, infra-red contact thermography with thermovision photographs (Mustakallio, 1979) and measuring skin thickness with Harpenden skin calipers (Kersey *et al.*, 1981). Kligman *et al.* (1967) reasons that the most

TABLE 2. Comparison of irritant threshold in five subjects before and after indomethacin

	MID% dithranol concentration	
Skin type	Before indomethacin	After indomethacin
I (Psoriasis)	0·1	0·5
I	0·1	> 1·0
I	0·2	> 1·0
II (Psoriasis)	0·2	> 1·0
III (Psoriasis)	0·3	> 1·0

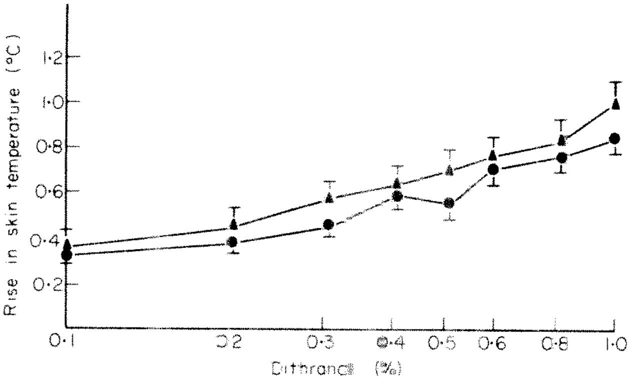

FIGURE 5. Inflammatory response to dithranol. Psoriatics (▲) and controls (●) (bars indicate standard error of the mean, $n = 40$).

discriminating feature of an irritant response is the threshold at which it occurs, as once the threshold has been exceeded the irritant response will not be the same in different subjects (Fig. 1). Our results differ from those of Kersey *et al.* (1981) in that we found there is no difference in irritant threshold between psoriatics and controls when all skin types are considered together. This may be the consequence of using different methods of assessing the irritant response. When skin thickness changes have been used as a measure of inflammation, as the test dose has been increased beyond the threshold for irritancy, the dose–response curves have been divergent and psoriatics were found more 'tolerant' of the inflammatory response (Kersey *et al.*, 1981). To test this we applied variance analysis to our data for the maximum temperature difference recorded at each site (Fig. 5) and found that the dose–response curves were parallel, but that psoriatics were less tolerant of dithranol irritation. However, differences between disease groups or skin types are no greater than differences between individual subjects. Probit analysis of each individual skin type confirmed the clinical impression that skin type I patients were more easily irritated than all other skin types but no significant differences were found between any of the other skin types. However variance analysis showed a 1 d.f. trend component representing a steady progression from type I to type IV (F ratio 5·93, $P < 0{\cdot}2$, Fig. 6). This trend component was also present when the irritant threshold was taken as 0·4°C, 0·5°C, 0·6°C, 0·7°C but not 0·8°C or 1·0°C.

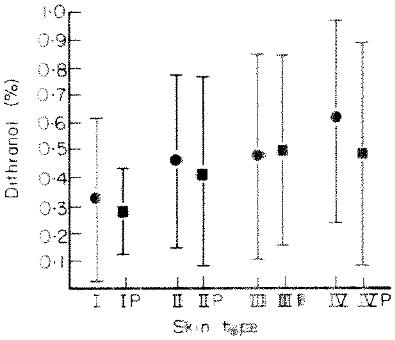

FIGURE 6. Concentrations of dithranol required to raise skin temperature by 0·5°C in different skin types. Controls (●) and psoriatics (■, P) Roman numerals refer to skin type.

We have shown that irritation and inflammation due to dithranol BP can be blocked by indomethacin, in contrast to previous reports (Misch *et al.*, 1981) where chromatographically pure anthralin was used. Indomethacin thus may be of value clinically in the treatment or prevention of dithranol erythema.

Our findings suggest that dithranol irritation is, at least in part, mediated by prostaglandins and can be blocked by inhibitors of the cycloxygenase pathway. It is of interest in this respect, that using the suction blister technique, increased levels of prostaglandins E have been found in dithranol irritated skin (Misch *et al.*, 1982).

Dithranol application remains one of the most useful types of treatment for psoriasis. It is surprising that comparatively little attention has been devoted to the irritancy that it can cause and which can impede or prohibit its use in some patients.

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The quantification of skin transparency

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SUMMARY

A new method for quantifying the transparency of skin using an adapted pair of Harpenden's Calipers is described. A constant for each skinfold's ability to transmit light, the H factor, is calculated and this shows a steady decline with age.

Transparent skin is commonly seen in elderly people. The main feature (McConkey *et al.*, 1963) is that the details of the veins can easily be seen. Both large and small veins have definite edges. The columns of blood within them are clearly visible and can easily be interrupted by light pressure. In opaque skin only the large veins are obvious and even when prominent their edges are not distinct. Transparent skin is not necessarily thin skin and although its aetiology is unknown it has been related to osteoporosis (McConkey, *et al.*, 1963). Attempts have been made to classify the skin either visually or using infra-red photography into three groups—transparent, opaque and doubtful. This paper reports a modification of Harpenden's Calipers, which have previously been used to measure skinfold thicknesses (McConkey *et al.*, 1963; Hall *et al.*, 1981) so that simultaneous readings of thickness and transdermal light transmission can be made.

METHODS

Harpenden's Calipers were modified by incorporating into one jaw a 5 V bulb which was controlled by a 43 Ohm resistor and 4·7 V zener diode powered by a 9 V battery, giving a constant light source. The amount of light transmitted was converted to an electrical voltage by a M.S.9 miniature silicone photo-voltaic cell incorporated into the other jaw and connected to a 100 A movement voltmeter. 5 K morganite pot. type 80 rheostat was connected in series. Simultaneous readings of skinfold thickness and the percentage of light transmitted can thus be measured.

Before each measurement the rheostat was used to adjust the intensity of the light source so that with the jaws closed the scale read 100%. The thickness and the percentage of light

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transmitted through the longitudinal skinfold over the fourth metacarpal of the left hand were measured with the hand at rest on a flat surface (Fig. 1). The readings were taken three times and the means calculated. Each reading was reproducible to within 4% (one division on the scale) for the light transmission and to within 0·2 mm for skin thickness. Any readings outside this range were scrutinized and found to be due to the trapping of subcutaneous tissues such as large veins between the caliper jaws. The skin was also visually classified into the three classical categories, transparent, opaque and doubtful. People taking steroids and those with rheumatoid disease or with dermatological problems affecting the hands were excluded.

The intensity of light in the same medium varies inversely with the square of the distance from the light source. A constant for each skinfold (which we have termed the H factor) can be calculated knowing the intensity of the light source, always 100%, the thickness of the skinfold and the percentage of light transmitted; i.e. H factor $= [(\text{skinfold thickness})^2 \times 100)/(\text{Percentage light transmitted})]$.

The H factor will differ with each skinfold. It will be independent of skinfold thickness and will give a value for the ability of light to pass through the individual skinfolds.

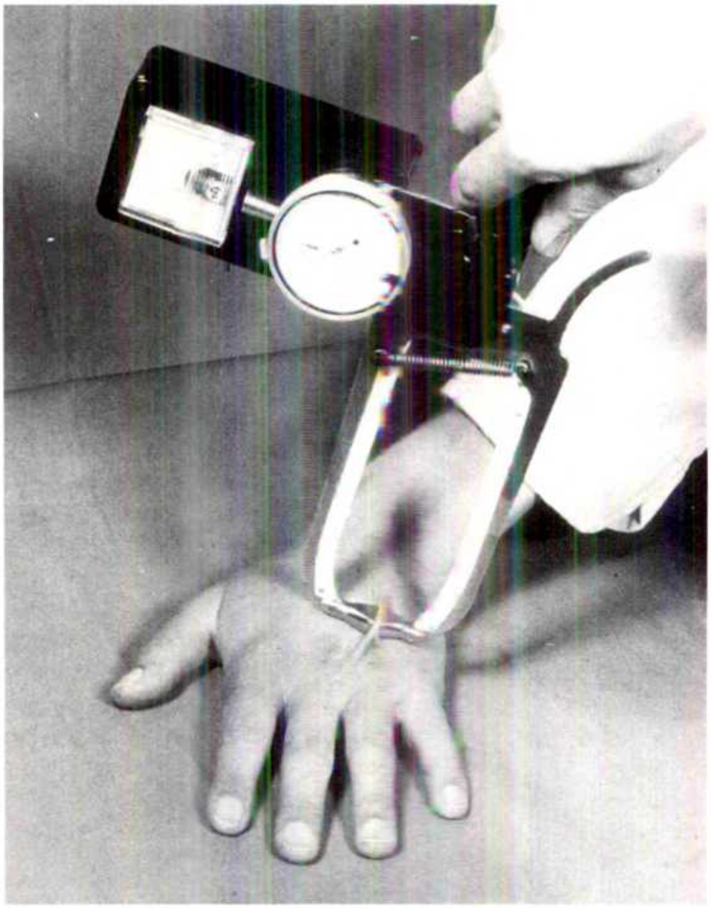

FIGURE 1. Photograph showing the modified calipers in use.

RESULTS

Skinfolds over the fourth metacarpal of 300 women aged 19–24 and 52 men aged 22–96 were measured.

There was a steady decline in skinfold thickness from the fifth to ninth decades in both sexes (Fig. 2). In the female group the values declined from a mean 2·6 mm ± 0·3 in the third decade to a mean 1·2 mm ± 0·25 in the ninth, the corresponding male values being 2·9 mm ± 0·2 and 1·35 mm ± 0·35. Throughout these age ranges males had thicker mean skinfolds than age-matched females, although there was considerable overlap between comparable groups. Thick skin tended to be opaque skin but it did not necessarily follow that thin skin was transparent (Fig. 3).

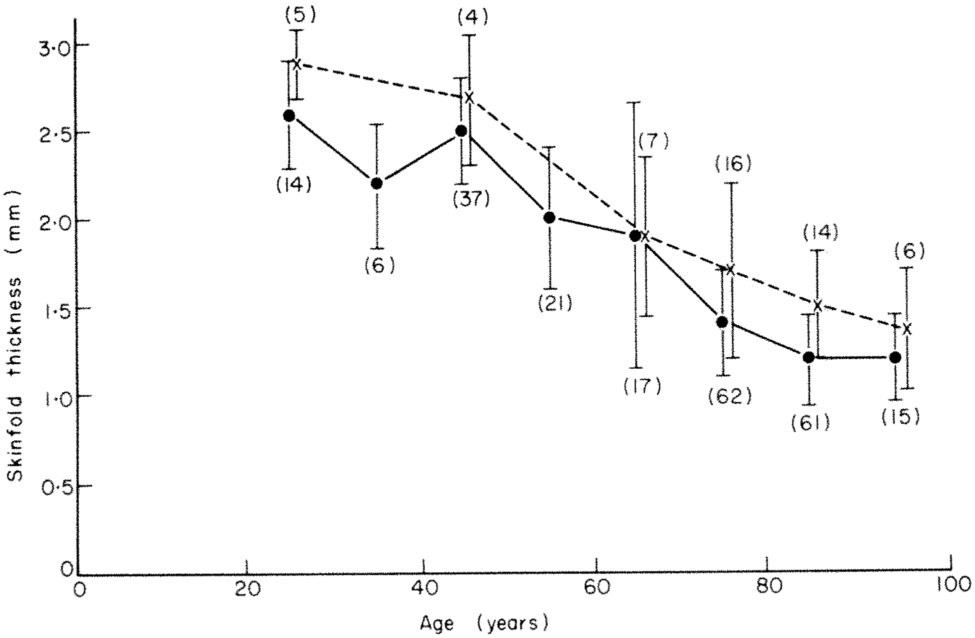

FIGURE 2. The relationship between skinfold thickness and age plotted in 10 year age groups, showing mean values for those age groups ± s.d., in female (●——●) and male (× – – – ×) subjects. Total numbers are in brackets.

Between the third and sixth decades there was no appreciable difference in the percentage of light transmitted through the skinfolds, the average value for light transmitted being 24 (Fig. 4). However, from the sixth decade onwards there was a steady increase in the mean values of light transmission but there was a large divergent scatter with some elderly people having similar values to those found in younger age groups, whilst others had a greatly increased transmission of light. The mean values of light transmission increased progressively from the seventh decade onwards. Transparent skin was only apparent clinically when the percentage of light transmission was above 29. No one under the age of 60 had transparent skin.

The mean value of the H factor declined steadily through the decades (Fig. 5). In the third decade the mean value for the female group was 34·5 ± 12 whilst in the 90-plus female age group it was 4·1 ± 3·5. The sudden divergence seen (Fig. 4) when only the percentage of light

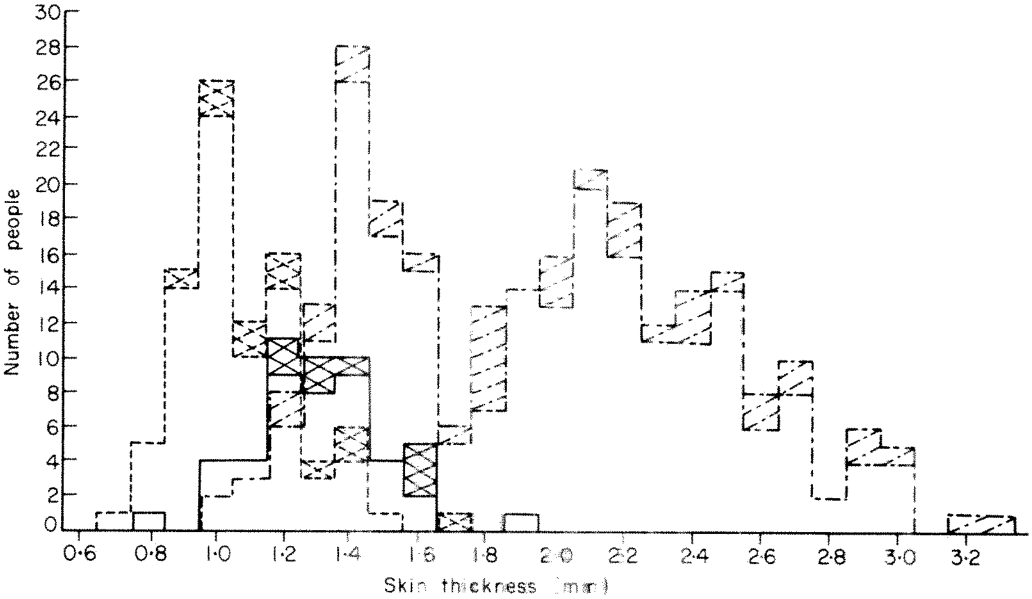

FIGURE 3. Histogram showing the number of subjects falling into 0·1 mm groups for the clinical subdivision of transparent (– – – –), doubtful (——) and opaque (–·–·–) skin. Hatched areas refer to males, unhatched areas to females.

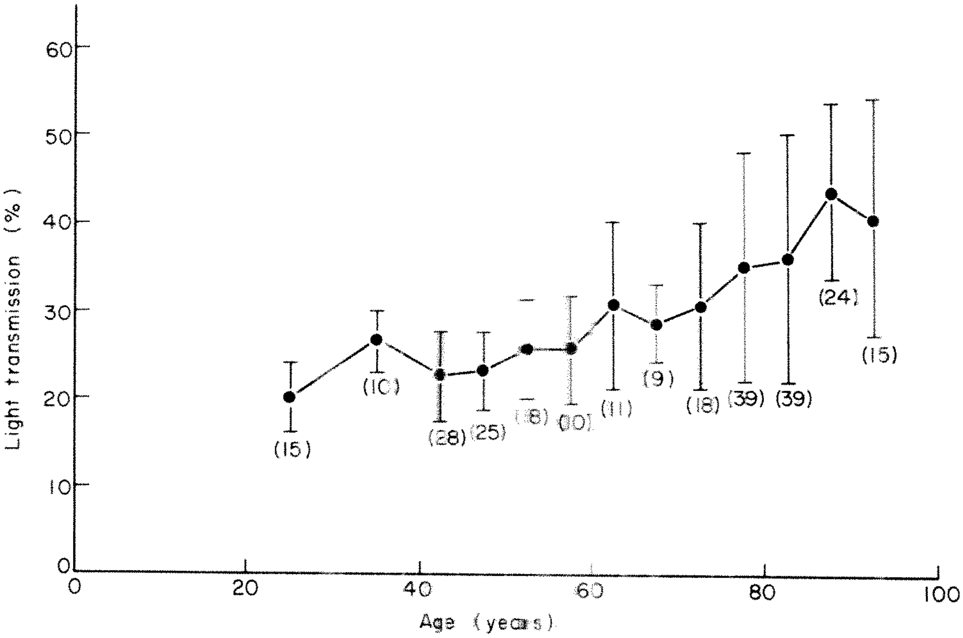

FIGURE 4. The relationship between percentage of light transmitted (mean ± s.d.) and age in female subjects. Total numbers are in brackets.

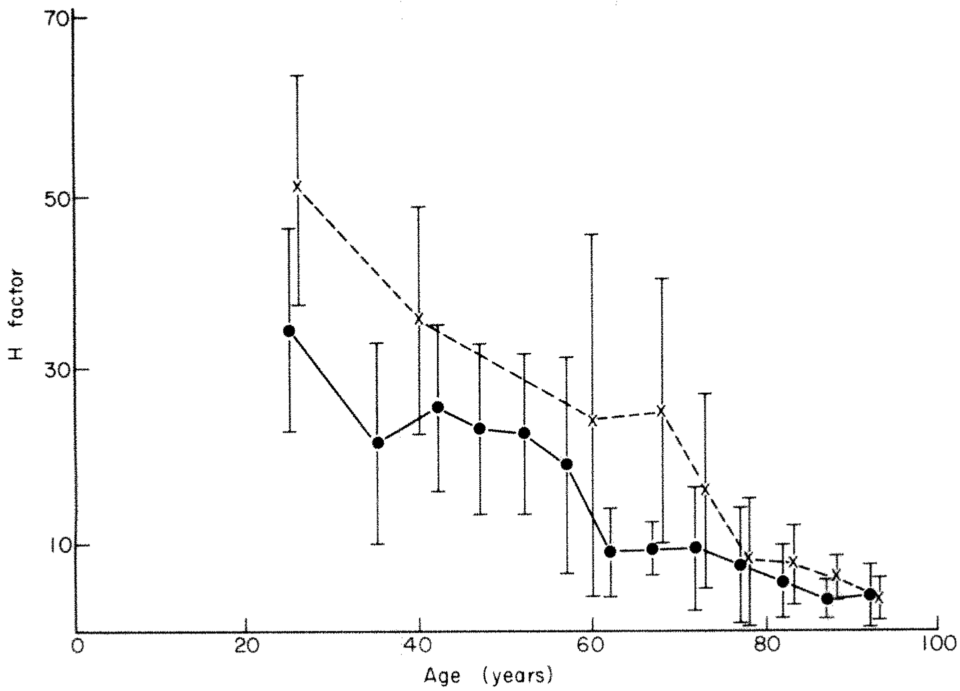

FIGURE 5. The relationship between H factor (mean ± s.d.) and age in males (×---×) and females (●——●).

transmitted was measured was not seen with the H values, which take skin thickness into account (Fig. 5) and the scatter of results, instead of becoming divergent, became more compact with age. Males have higher H values than age-matched females up to the seventh decade when they become similar. The mean value in the third decade for the male group was 50·5 ± 13 whilst in the 90 plus age group it was 3·6 ± 2·3.

DISCUSSION

In both sexes transparent skin tends to be thick but thin skin is not necessarily transparent and there is a large overlap between the skinfold thicknesses of the two main clinical groups, 'opaque' and 'transparent' with the 'doubtful' group overlapping both of these (Fig. 2). It is confirmed that skinfold thickness diminishes with age (Hall *et al.*, 1981). In early adult life the decline is only very slight but it increases after the fourth decade. This pattern is seen in both males and females, although after 90 years of age the decline is lost in the female but continues in the male. The calculation of the H factor, which takes into account the thickness of the skinfold, shows that this also steadily declines with age in both males and females, although it is not until the seventh decade that the mean values are similar for both sexes.

Transparency seems therefore to be associated with some intrinsic change in the skin itself. This is unlikely to be due to changes in fat for unless there is gross obesity the amount of subcutaneous fat on the dorsum of the hand is small in amount and fairly constant (McConkey *et al.*, 1963). It is therefore likely to be due to a change in the dermis, and dermal collagen is known to change with age. The amount of dermal collagen and skin thickness both diminish with age, although the former does so to a greater extent thereby producing a diminution in collagen density (Shuster, Black & McVitie, 1975).

Is there in addition a change in the structure of the collagen itself which makes light pass through it more easily, perhaps in the same way that light is transmitted through the cornea as opposed to the sclera? McConkey *et al.* (1963) suggested that transparent skin is associated with osteoporosis; and the use of this instrument makes it possible to compare light transmission through skin with radiological examination of bone. Further work is needed to identify whether transparent skin is solely a cutaneous ageing phenomenon, perhaps exacerbated by exposure to sunlight, or is associated with a concomitant age change in collagen elsewhere in the body. If this is so then the use of this instrument could give an easy non-invasive way of measuring skin and collagen ageing.

ACKNOWLEDGMENT

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Effect of castration and testosterone propionate on mouse vibrissae

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SUMMARY

Vibrissae of normal mice were measured for six cycles starting at birth. Groups of immature male albino mice were castrated at 21 days of age when E, F, G and H (Danforth nomenclature, 1925) vibrissae were in their first cycle of growth. Levels of testosterone in their peripheral plasma were reduced to about one tenth of the normal. The final length achieved by their vibrissae in all subsequent cycles was shorter than that of normal mice. Another group of immature male mice were treated with implants of testosterone propionate to give plasma levels of approximately five to ten times normal. Their vibrissae showed a significant increase in final length in all growth cycles before and after sexual maturation.

Vibrissae of adult male mice treated with excess testosterone showed a significant increase in final length at the end of the growth cycle immediately after commencement of treatment. On the other hand, vibrissae of castrated adult mice showed a significant decrease in final length in the second successive cycle after castration.

The rate of growth of all vibrissae remained unchanged. Changes in their final length were brought about by changes in the period of growth. Castrated mice had a slightly longer resting period.

Although androgens have been shown to stimulate hair growth in different laboratory animals and man (Hooker & Pfeiffer, 1943; Mohn, 1955; Johnson, 1958; Papa & Kligman, 1965; Guarrera *et al.*, 1976) their influence on the specific characteristics of the hair (final length, growth rate, hair width, etc) have been difficult to estimate. In this study we report the growth of vibrissae in male mice which received treatments to alter the androgen concentration in their peripheral plasma. It is suggested that this technique provides a simple and accurate approach to the study of such endocrine effects on hair growth.

Randomly bred albino mice were used. The posterior column of four whiskers named E, F, G and H were chosen because they are the largest and easiest to locate for measurement (Ibrahim &

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Wright, 1975). These whiskers are 2–3 mm long when the mice are born and in healthy young mice they grow 1 mm/day for about 3 weeks in the first cycle. The rate of growth slows down for a few days before reaching a final length of 25–30 mm. The new whisker (of the second cycle) starts growing while the old one has stopped growing but is still present in the follicle. About 10–15 days later when the new whisker is approximately 10–15 mm long the old one falls out. This has no effect on the growth of the new vibrissa (Ibrahim & Wright, 1975; 1978). Each follicle behaves independently of adjacent follicles and the vibrissa produced has a specific rate of growth, length of cycle, constant resting period and final length (Ibrahim & Wright, 1975).

The effect caused by any applied factor can be detected and measured easily. The only change in the final length of the whiskers occurs exclusively in male mice. This is an increase of about 2–3 mm, taking place during the third cycle, which commences at 9 weeks of age and of 0·5–1 mm in the fourth. Since this main increase in length occurs in the cycle immediately after the mice reach sexual maturity at 6 weeks of age, the experiments were designed to find whether this increase is linked with the increase in the levels of sex hormones which takes place at maturity and if so, could it be prevented by early castration and enhanced by early introduction of testosterone?

Also fully mature 3-month-old mice were used to find the effect of the same hormonal treatments on the growth of their vibrissae. Vibrissae of intact female mice were also measured and included.

MATERIALS AND METHODS

Testosterone propionate

Testosterone propionate (TP) was supplied by Sigma Chemical Company (Poole, Dorset) as a powder and was compacted into pellets of 20–40 mg. In some experimental groups these were implanted subcutaneously on the back of mice. This pellet of pure TP was found, by weighing, to dissipate at the rate of 0·2 mg/day (the same figure was reported by Ebling & Johnson, 1964, using testosterone). A new pellet was implanted after 90–100 days.

Levels of testosterone in the blood were measured by radioimmunoassay, after purification of the analyte by column chromatography. Manipulative losses were assessed and corrected by addition of tritiated testosterone. The precision of the assay (as coefficient of variation) was 10%. Testosterone in the blood (nmol/litre) was estimated for normal and treated mice at the beginning of, at various times during, and at the end of, the experiment (Table 1).

Whisker measurement

All mice had their whiskers (E, F, G and H on both sides) measured weekly for three or four cycles. All measurements were carried out under a dissecting microscope at a magnification of sixteen times and white whiskers were dyed using methylene blue or crystal violet to aid measurement. The whisker was located, cut off (at the time of first measurement) and stuck to a microscopic slide for later measurement. The remaining stump was measured by placing over it a calibrated capillary glass tube with 0·1 mm divisions and pushing gently against the skin. The two measurements were added to make the total length of the whisker. The stump was then measured repeatedly during the cycle. Measurements were made to the nearest 0·1 mm and could be repeated by different observers (Ibrahim & Wright, 1975).

Animals

Two batches of albino mice were used. (A) Twenty 21–24 day old male albino mice (i.e. 5–9 days

TABLE 1. Effects of castration or implantation of testosterone propionate at 3 weeks of age on plasma testosterone concentrations in male albino mice

Treatment	No. of mice†	Age (days)	Mean concentration of testosterone	Range	Mean body weight (g)
Normal	4	21	4·12	3·16–5·44	12·50
	4	28	12·11	4·56–21·05	17·60
	6	78–84	11·68	3·09–31·05	42·10
Castrated	2	30	0·75	0·42–1·09	29·00
	4	45	0·35	0·35–0·35	31·90
	2	110	0·45	0–0·95	37·25
	4	120	1·38	0·35–21·05*	30·75
	12	210–225	0·72	0–0·98	42·50
Intact + testosterone	3	30	139·29	78·95–181·05	27·70
	2	120	68·77	60·35–77·19	29·25
	4	210	81·67	57·89–102·46	38·40

* One reading of 21·05 was reported in a mouse which was not used for whisker measurement. As this was so discordant with all the other findings, it has been excluded when calculating the mean.

† For technical reasons a number of samples were not suitable for analysis.

Values are in nanomoles per litre.

before commencement of the second cycle of whisker growth) were divided into three treatment batches: (i) eight castrated; (ii) six normal intact; (iii) six intact + testosterone. Six 21-day-old intact female mice were also used. (B) Eighteen fully mature, 3 month old male mice were divided into (i) six castrated; (ii) six intact control; (iii) six intact + testosterone. Six 3 month old, intact female mice were also used.

Mice of group A were killed at the end of the observation period (120–225 days) and levels of their plasma testosterone were estimated (Table 1). In order to assess the effect of treatments at early periods on the concentration of plasma testosterone a further group of thirty-six male mice, 3 weeks old, were divided into three treatment batches: ten control, twenty castrated and six intact + testosterone. A few of each batch were killed at various intervals during the experiment and the testosterone in the blood was estimated and included in Table 1.

RESULTS

Subcutaneous implantation of testosterone propionate into mice increased the hormonal level in their peripheral plasma to many times that of normal intact male mice. On the other hand, levels of the hormone in peripheral blood of castrated mice was reduced to a small fraction of that of the normal (Table 1). The final length of the animal's vibrissae always increased after testosterone treatment. After castration the vibrissae of the immature animals failed to increase significantly while in the mature animals the vibrissae decreased significantly (Fig. 1). Vibrissae of the normal immature male mouse followed the normal pattern of growth described earlier. They were of similar final length at the end of the first and second cycles and increased by 2·5 mm at the third cycle (puberty) and 1 mm at the fourth cycle. They reached 27·64 mm ± 0·25, 28·05 mm ± 0·46, 30·64 mm ± 0·32 and 31·53 mm ± 0·29 in their four successive cycles of growth. Statistical

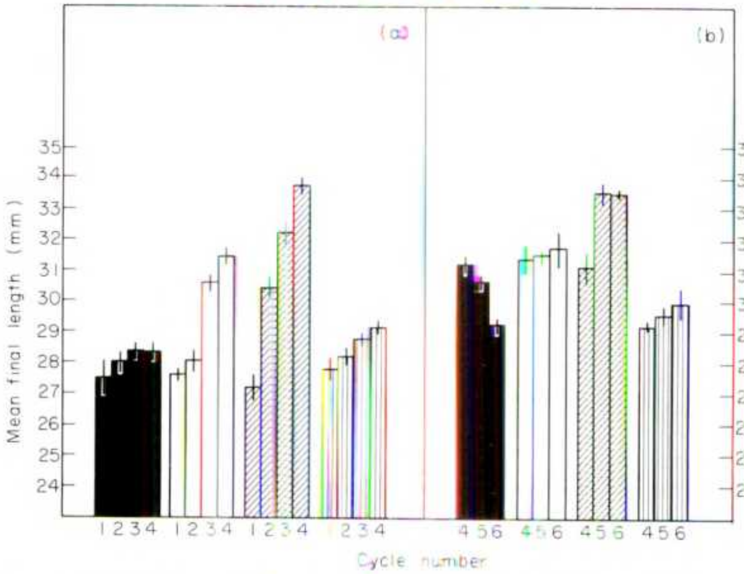

FIGURE 1. Mean final length ± s.e. of vibrissae of albino mice measured for three or four successive growth cycles in the different groups of treatment; normal intact, castrated and testosterone treated male mice. Vibrissae of female mice are also included. (a) Young immature mice, 21–24 days old, measurements started at the first cycle of vibrissal growth. (b) Young adult mice 3 months old, measurements started at the fourth cycle of vibrissal growth. Castrated (■); normal male (□); with excess testosterone (▨); female (▩).

analysis of the results showed that the increase in the whiskers' final length at the end of the third cycle was significant ($P < 0{\cdot}005$).

At the end of the second cycle (before sexual maturation) the vibrissae of immature testosterone-treated male mice were on average 3·2 mm longer than those of normal males and not significantly different from normal third cycle whiskers. They were also 2 mm longer at the end of the third cycle and 1·5 mm at the end of the fourth (Fig. 1a; first cycle = 27·19 mm ± 0·45; second cycle = 30·44 mm ± 0·37; third cycle = 32·27 mm ± 0·38; fourth cycle = 33·85 mm ± 0·33). Statistical analysis (*t* test) showed the increases to be highly significant at the second and third cycles. When statistical tests were carried out comparing vibrissae of intact mice with those of TP-treated mice, the TP treated were found to be significantly longer at the end of their second, third and fourth cycles ($P < 0{\cdot}005$).

Vibrissae of castrated, immature mice were almost the same final length for four successive cycles (27·5 mm ± 0·48; 27·97 mm ± 0·33; 28·48 mm ± 0·27 and 28·40 mm ± 0·25). They were significantly shorter than those of normal control mice at the end of the third and fourth cycles ($P < 0{\cdot}005$).

At the end of the fourth growth cycle, vibrissae of castrated mice were slightly, but not significantly, shorter than those of their intact female counterparts (Fig. 1a).

Vibrissae of female mice followed a consistent pattern of growth for 7 months starting at 3 weeks of age. They showed a gradual increase averaging 0·4 mm in every successive cycle (Fig. 1a and b first cycle = 27·81 mm ± 0·44; second = 28·23 mm ± 0·33; third cycle = 28·88 mm ± 0·23; fourth cycle = 29·2 mm ± 0·21).

Vibrissae of normal mature male mice were nearly of the same final length at the end of the

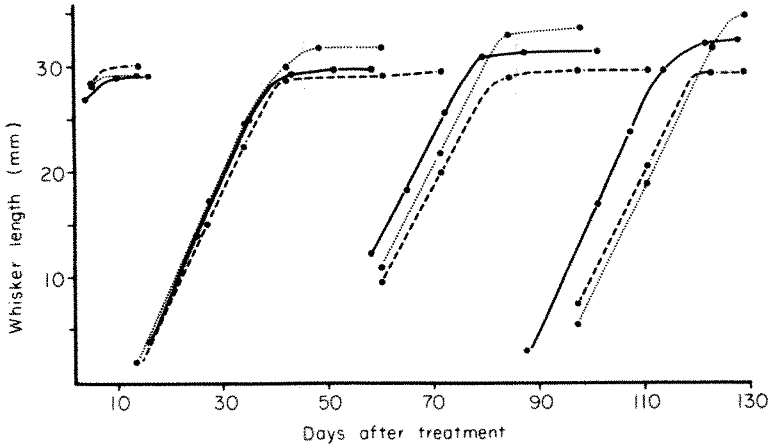

FIGURE 2. Lengths of a single G vibrissa to illustrate the growth of normal intact albino mice vibrissae (——), of intact, testosterone-treated mice vibrissae (– – –) and of castrated mice vibrissae (· · · · ·) at the end of the first cycle of growth and during the subsequent second, third and fourth cycles. Whilst the terminal length of vibrissae varied in all but the first cycle the rate of growth and time of initiation remained unchanged.

three successive cycles measured (Fig. 1b). They were: 31·47 mm ±0·54, 31·72 mm ±0·40 and 31·77 mm ±0·66, successively.

Vibrissae of testosterone-treated adult male mice were about 2·5 mm longer at the end of their growth cycle immediately after treatment (the increase was statistically significant, $P = 0{\cdot}050$). This was maintained during the next cycle; they were 30·72 mm ±0·68, 33·01 mm ±0·51 and 32·98 ±0·22, successively.

Vibrissae of castrated adult mice lost a total of 2 mm over the two growth cycles following castration, at the end of which they were slightly (but not significantly) shorter than vibrissae of female mice of the same age (Fig. 1b). Vibrissae of adult female mice continued the same pattern of growth as that of young mice (Fig. 1a and b). They were 29·25 mm ±0·22, 29·58 mm ±0·32, 30·02 mm ±0·53 at the end of the three successive cycles.

Although increases and decreases in the vibrissal final length were achieved by applying the different hormonal treatments, their rate of growth was not affected (Fig. 2). Vibrissae of castrated animals had a slightly longer telogen, while those of testosterone treated mice had a slightly shorter telogen phase (Fig. 2).

All mice were weighed at various times during the experiment (Table 1). Increases and decreases in the vibrissae final length were not related to body weight.

DISCUSSION

Mouse whiskers have proved to be very satisfactory for the study of the effects of hormones because their normal growth characteristics are extremely constant and small variations can be measured easily and accurately. Vibrissae of mice of both sexes grow to the same final length in their first two cycles. In the third cycle the male vibrissae are 2–3 mm longer and there is a further increase of another 1 mm in the fourth cycle (Ibrahim & Wright, 1975). In the experiments carried out here it was evident that the increase in length was due to the increase in the hormonal level in the male mouse. Animals castrated before adulthood continue to have short whiskers for at least 6 months. These whiskers are approximately the same length as or slightly shorter than the female whiskers. The male, however, with an excess of testosterone early in life showed the growth of a few extra millimetres taking place in the second cycle as opposed to the third. Guarrera *et al.* (1976) found that the addition of increasing concentrations of testosterone to the medium of rat embryo skin explants in culture provoked an increase in volume and better differentiation in the hair follicles. We found that the vibrissae of mice treated early reached 34 mm in length. This was 13% more than the final length reached by vibrissae of the normal intact male.

The effect of these treatments on adult mice is slightly different. Excess testosterone had an immediate effect on the whiskers' final length which was maintained at the next cycle, while castration was not very effective on the growth cycle immediately afterwards but had a significant effect on the second successive cycle.

It is interesting to find that although large individual animals have longer vibrissae, castrated animals were slightly heavier than their control counterparts, but their vibrissae were shorter.

When testosterone was applied after the start of a cycle it did not affect the growth of that cycle. Testosterone caused an increase in whisker length and castration resulted in the reverse. The rate of growth and initiation time were normal in all groups of treatment.

ACKNOWLEDGMENTS

We are pleased to thank Professor V.H.T. James of St Mary's Hospital, London, for the estimation of the testosterone levels in the blood.

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Pharmacology and Treatment

Treatment of rosacea with 1% metronidazole cream. A double-blind study

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SUMMARY

Eighty-one patients with rosacea were treated with either 1% metronidazole cream or the cream base as a placebo for two months. The trial was performed double-blind, and the patients were assessed once each month. The variates studied were: (1) overall clinical assessment, (2) lesion counts, (3) degree of erythema, (4) independent photographic evaluation, and (5) patient's opinion.

Four patients dropped out of the trial (one treated with metronidazole, three with placebo). In all the variates, 1% metronidazole cream proved to be significantly more effective than placebo.

Metronidazole has previously been administered systemically to patients with rosacea, and the effect has been shown to be significantly better than placebo, and comparable to results achieved with tetracyclines (Pye & Burton, 1976; Saihan & Burton, 1980; Guilhou *et al.*, 1978; Stieger, 1978). Therefore, it was considered of interest to test a topical preparation of metronidazole. In an open study in fourteen patients with rosacea, promising results were achieved with 1% metronidazole cream (Gamborg Nielsen, 1982). To verify these results a double-blind study versus placebo has now been performed.

METHODS

Eighty-one randomly selected patients (forty-nine women, thirty-two men) with an average age of 47 years (range: 26–87 years) suffering from rosacea of differing degrees of severity entered the study. The trial was performed as a double-blind study using 1% metronidazole cream versus placebo.

Since the intensity of rosacea fluctuates during the year, due to the northern climate, it was important to perform the study during a limited period of time. For all visits to the clinic we followed a procedure whereby the patients were studied during the same week. The trial was performed during January and February of 1982.

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Patients were provided with 50 g of the test cream which they applied once daily over a period of two months. The cream base used for both the placebo and the metronidazole cream was an oil in water emulsion (lactic acid 1·5%, sodium lauryl sulphate 0·8%, cetylane 5%, cetanole 15% and distilled water 80%). The average amount of cream applied to the face during the study period was 31·4 g (range: 12·0–51·4 g). The mean amount of metronidazole applied by each patient was 3·75 mg a day (range: 2·17–6·82 mg). The treatment area was limited to the face. None of the patients had been treated for rosacea in the three months preceding the trial.

Patients were assigned to one of the two courses of treatment in accordance with a randomized administration scheme. Forty-one were allocated to 1% metronidazole cream, whilst forty received the cream base. None of the patients used any other kind of treatment during the study period.

Each patient underwent a clinical evaluation and was photographed prior to the study.

Follow-up examinations were performed after one and two months treatment. These included: (1) clinical assessment, (2) lesion counts, (3) assessment of erythema compared with a colour scale ranging from 1–5, (4) number of telangiectasia, (5) standardized photography together with the colour scale, and (6) patient's subjective opinion of treatment effect.

Nos. 1 and 5. The objective clinical assessment was expressed in the four category acne classification of Pillsbury, Shelley & Kligman (196[illegible]), as was the photographic evaluation which was performed by two independent dermatologists (Table 1).

No. 2. The papules and pustules were counted at each visit (Table 2).

No. 3. Reduction of erythema was expressed as the sum of the difference of the colour range at every visit (Table 2).

No. 4. Telangiectasia were expressed by area, + referring to the nose, + + the nose and infraorbital region, and + + + the nose, infraorbital region and other areas of the face.

No. 6. The patient's subjective opinion of treatment results was classed as much improved, improved, somewhat improved, unchanged, worse or much worse (Allen, 1980) (Table 3).

The overall clinical and photographic assessments were fundamental for the documentation of the therapeutic effect. The method of photography was standardized for all patients. In order to compensate for variations in film emulsions, light intensity, aperture, background and distance a special colour range was performed consisting of five different lesion colours of which numbers 1 and 2 were different shades of normal skin colour. Numbers in the colour range refer

TABLE 1. Degrees of improvement in seventy-seven patients after treatment with 1% metronidazole cream and placebo. The results are expressed as percentage improvement according to the photographic and clinical findings

	Metronidazole				Placebo			
	1 month		2 months		1 month		2 months	
Degree of improvement	Photo.	Clinical	Photo.	Clinical	Photo.	Clinical	Photo.	Clinical
0–25%	12	10	12	12	25	23	21	24
26–50%	3	4	2	4	1	4	0	5
51–75%	9	5	6	5	5	3	9	0
76–100%	16	21	20	19	6	7	7	8
Total patients	40	40	40	40	37	37	37	37

TABLE 2. Total and mean values of erythema, papules and pustules before treatment and after 1 and 2 months' treatment

	Before		1 month		2 months		*P* value at 2 months
	Total	Mean	Total	Mean	Total	Mean	
ERYTHEMA							
Clinical (metronidazole)	150	3·8	115	2·9	100	2·5	<0·05
Clinical (placebo)	136	3·7	128	3·5	116	3·1	
Photographic (metronidazole)	143	3·6	114	2·9	107	2·7	<0·05
Photographic (placebo)	127	3·4	108	2·9	105	2·9	
PAPULES							
Metronidazole group	951	23·8	308	7·7	344	8·6	<0·05
Placebo group	1016	27·5	536	14·5	615	16·6	
PUSTULES							
Metronidazole group	22	0·6	8	0·2	12	0·3	<0·05
Placebo group	37	1·0	31	0·8	30	0·8	

TABLE 3. Subjective opinion of treatment results, expressed in a six category classification, in metronidazole and placebo groups

	Metronidazole		Placebo	
	1 month	2 months	1 month	2 months
Much improved	12	16	2	2
Improved	10	9	7	6
Somewhat Improved	9	4	6	5
Unchanged	9	11	19	21
Worse	0	0	3	3
Much worse	0	0	0	0
Total	40	40	37	37

to the most pronounced area of erythema. The colours used in the range were selected from Alcro 3D system, Sweden, with the following colour symbols: 1) R3Y10, 2) R1Y30, 3) R51, 4) R1B52, 5) R1B53. This colour scale was used to assess changes of erythema at the follow-up examinations (methods for assessment of treatment effect will be published later as a methodological study concerning rosacea).

Side-effects were registered. Prior to the study 100 patch tests (fifty metronidazole cream and fifty cream base) were performed. At the end of the study all patients were tested with 1% metronidazole cream using the method recommended by the International Contact Dermatitis Research Group.

Patients were investigated for blood levels of metronidazole both before the trial and after one month's treatment. For the statistical calculations of results the chi-square test was used.

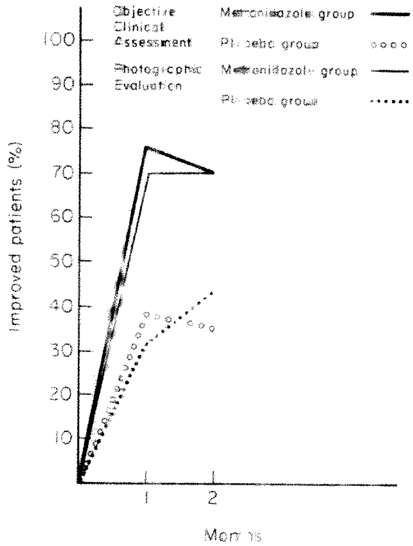

FIGURE 1. Improvements in the metronidazole and placebo groups, expressed in percentages, and assessed through objective clinical estimation and photographic evaluation.

RESULTS

The number of patients who showed improvement on 1% metronidazole cream versus placebo is seen in Fig. 1. After both one and two months' treatment, improvement in the metronidazole group was significantly greater than in the placebo group ($P < 0{\cdot}05$). The photographic and clinical degrees of improvement expressed in a four-category classification are shown in Table 1. Reduction of redness, papules and pustules in the metronidazole and placebo groups (expressed as total and mean values) are shown in Table 2. Patient's opinion is shown in Table 3.

The extent of telangiectasia was unchanged during the study period.

Blood metronidazole levels were assayed in patients treated with 1% metronidazole cream. Traces of metronidazole were found in ten of forty patients using the HPLC method (Hackett & Dusci, 1979; Arnold, 1982). Results ranged from < 20–45 ng/ml.

In 100 patch tests performed prior to the study eleven irritant reactions were found (five caused by metronidazole cream and six by the cream base). In the group of seventy-seven patients who completed the trial, nine slight irritant reactions were observed when they were tested with the substance with which they had been treated (four caused by metronidazole cream and five by the cream base). However, these had no influence on the treatment results. Four patients left the study (one from the metronidazole group and three from the placebo group) after one month's treatment owing to an absence of improvement.

No patients reported allergic or irritant reactions in the treatment area but the cream had a drying effect on the skin of most patients, probably due to the vehicle.

DISCUSSION

The accepted treatment for rosacea is systemic tetracycline, which improves 80–90% of patients. Comparable results are found in patients treated with oral metronidazole (Saihan &

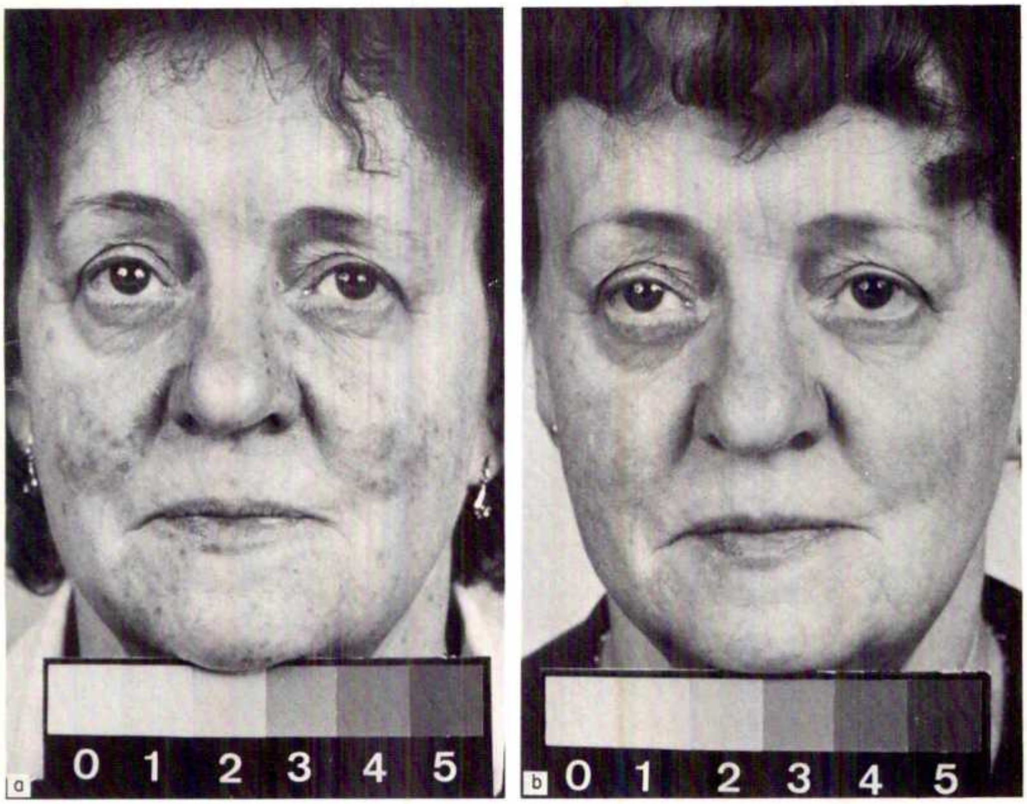

FIGURE 2. Photographs of patient (a) before application of 1% metronidazole cream, and (b) after 2 months' treatment.

Burton, 1980). Topical metronidazole was tried in 1980 (Prinz, 1980), but the improvement was not convincing, probably because metronidazole was not released sufficiently well from the vehicle. The second study on the topical use of metronidazole for rosacea was performed as an open study and showed improvement in 90% of the patients (Gamborg Nielsen, 1982) which is comparable to oral administration. In the first publication a 5% metronidazole cream was used and in the second a cream containing 1% metronidazole. This indicates that the composition of the vehicle was important for the release of metronidazole.

In the present study it was proved that 1% metronidazole cream was significantly better than placebo. The level of improvement was equal to results achieved in the open study, and compares favourably with orally administered metronidazole and tetracyclines (Guilhou *et al.*, 1978; Stieger, 1978).

Since the minimum dose of oral metronidazole for improvement of rosacea is about 200 mg a day (Guilhou *et al.*, 1978) the difference of blood level metronidazole from a single dose of 250 mg, and the average amount of metronidazole (3·75 mg/day) applied to the face was considered. Mean curve peaks of molecularly unchanged metronidazole within 1 h showed a maximum concentration of 4–7 μg/ml after oral administration, and 70 ng/ml after topical application. As the blood concentration of metronidazole during topical treatment is about one hundred times lower than that found during systemic treatment, the beneficial effects must be due to a local mode of action.

There is no known explanation for the effect of metronidazole on rosacea. None of those microorganisms, including *Demodex folliculorum*, which are found in rosacea lesions are sensitive to metronidazole (Höffler, Niederau & Pulverer, 1980; Persi & Rebora, 1980). The effect of metronidazole may relate to suppression of cell-mediated immunity (Grove, Mahmoud & Warren, 1977), the invasion of microorganisms being secondary to this process.

ACKNOWLEDGMENTS

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A dose–response study of 13-cis-retinoic acid in acne vulgaris

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SUMMARY

13-cis-retinoic acid has been used as a single agent in the treatment of seventy-six patients with previously unresponsive cystonodular acne. The study was carried out in a double-blind fashion using three doses of the drug. Ninety per cent of the patients responded with a 70% improvement in the acne severity. Sixty-six per cent of the patients experienced no further problems with their acne during follow-up. Side-effects were frequent. The 0·5 mg/kg b.w. dose is recommended for the initial course of treatment.

The conventional therapy of moderate to severe acne consists of long-term oral antibiotics, and is successful in most patients. However, there is a small but difficult group of patients who fail to improve. A synthetic derivative of vitamin A, 13-cis-retinoic acid, was reported (Peck *et al.*, 1979) to produce a dramatic improvement in this minority group. Further studies (Jones, Blanc & Cunliffe, 1980; Jones, Cunliffe & Cove, 1981; Farrell, Strauss & Stranieri, 1980; Plewig, Wagner & Braun-Falco, 1980) have confirmed these observations. This drug therefore has potential as an alternative form of therapy in acne. A double-blind dose–response study on seventy-six patients is now reported, the main aim of which was to determine the optimum dosage for clinical use.

METHODS

Seventy-six patients with moderate to severe acne were selected for inclusion in the study on the following criteria: (1) failure to respond to long-term oral antibiotic therapy; (2) partial response to antibiotics, but rapid relapse on cessation of such therapy.

The extent of the patients' acne was assessed under standard lighting conditions by: (1) grading on a 0–10 scale the severity of the condition on the face, back and chest (Cunliffe, 1981; Burton *et al.*, 1971); (2) counting the non-inflamed, superficial and deep inflamed lesions of the face.

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Sebum was collected over a 3-h period and was estimated by a gravimetric method (Strauss & Pochi, 1961), thus enabling the sebum excretion rate (SER) to be calculated.

Previous therapy for the patients' acne was discontinued 6 weeks prior to the start of the trial. Because of the strict selection criteria, patients were recruited over an 11-month period (February 1980 to January 1981). Informed consent was obtained from each patient, with emphasis being placed on the need for adequate contraception. Three of the thirty-one females were already taking a contraceptive pill, and a further three started it at the beginning of the trial.

After the initial visit, the patients were seen at 4-week intervals during a treatment period of 16 weeks, and a follow-up period of 16 weeks. At all visits, SER and acne severity were estimated, and side-effects noted.

Patients were divided randomly into three groups on a double-blind basis for treatment with 0·1 mg, 0·5 mg and 1·0 mg per kg of body weight (b.w.) of oral 13-cis-retinoic acid (Table 1). No other therapy for the acne was prescribed.

TABLE 1. Patient treatment groups (mean ± S.E. values)

Dose group (mg/kg b.w.)	0·1	0·5	1·0
Patient numbers	22	30	24
Male	11	17	17
Female	11	13	7
Age (years)	23·7 ± 1·1	24·3 ± 1·3	24·6 ± 1·7
Sebum excretion rate ($\mu g \cdot cm^{-2} \cdot min^{-1}$)	1·74 ± 0·17	1·90 ± 0·15	1·95 ± 0·20
Acne grade	4·60 ± 0·77	4·87 ± 0·50	7·35 ± 0·99
Acne lesions	54 ± 10	54 ± 9	79 ± 17

The parametric data (SER and acne lesion count) were analysed by Student's *t*-test for changes within the groups, and by one-way analysis of variance for comparison between the groups. Wilcoxon's rank sum test was used for analysis of the non-parametric data (acne grade).

RESULTS

During treatment

Sebum excretion rate (Fig. 1). The SER was reduced by 75% in the 0·1 mg/kg b.w. group, 89% in the 0·5 mg/kg b.w. group and 91% in the 1·0 mg/kg b.w. group. This reduction occurred predominantly within the first 4 weeks. The changes are highly significant ($P < 0{\cdot}0005$), and there is a significant difference between the effects of the low and high doses at 16 weeks ($P < 0{\cdot}05$).

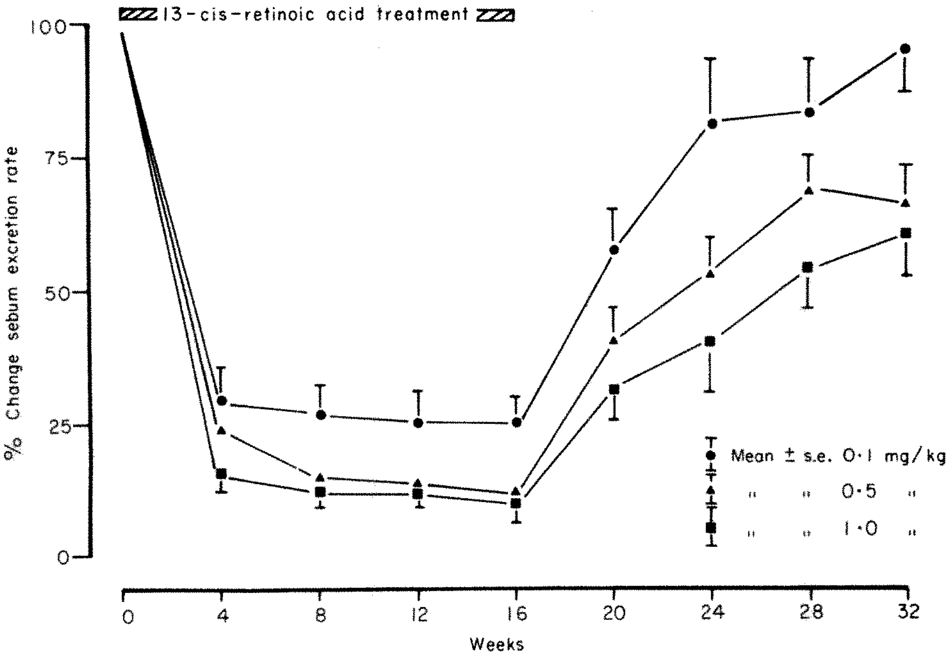

FIGURE 1. Change in sebum excretion rate during and off therapy.

Clinical response (Figs 2 and 3). There was an overall good clinical response in terms of both the acne grade and the acne lesion count gradually over 16 weeks. The acne grade showed a 70% improvement in the 0·1 mg/kg b.w. dose, and 65% improvement in the other two groups by the end of the treatment period ($P<0\cdot01$). There was a parallel reduction in the numbers of acne lesions of the face. By 16 weeks there was an 80% reduction in the 0·1 and 0·5 mg/kg b.w. groups, and an 89% reduction in the 1·0 mg/kg b.w. group ($P<0\cdot0005$).

These reductions are all statistically significant but there was no significant difference between the doses.

There appeared to be a differential effect of 13-cis-retinoic acid at the different sites of the acne. In the 1·0 mg/kg b.w. group, there was an 80% improvement in the acne of the face, and 54–56% improvement in that of the chest and back. This pattern was reflected in the other dose groups.

The numbers of the different types of acne lesions were reduced equally in all dose groups.

During follow-up

Sebum excretion rate (Fig. 1). The dose differences are more obvious during the follow-up period. At 32 weeks, the SER in the 0·1 mg/kg b.w. group has returned to 95% of the pre-treatment level, whilst in the other two groups there is only a return to 60–66% of those values. This difference between the low and other groups at 32 weeks is significant ($P<0\cdot05$), and the SER in the high dose groups is still significantly different from the pre-treatment value ($P<0\cdot0025$).

Clinical response (Figs 2 and 3). The effect of 13-cis-retinoic acid was to produce a clinical

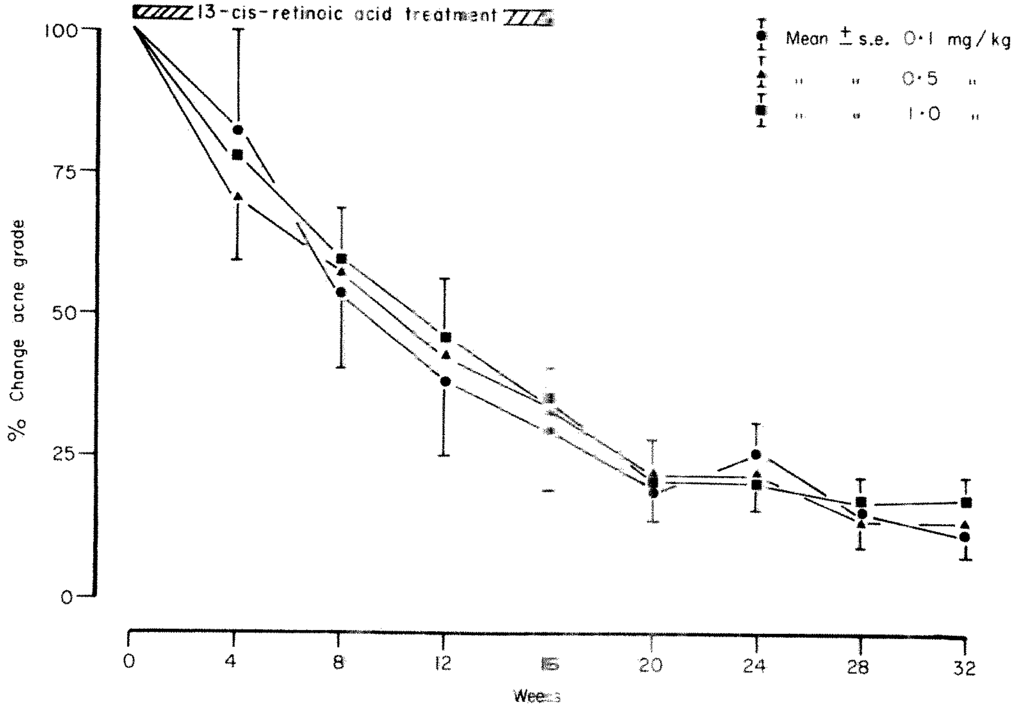

FIGURE 2. Change in acne grade during and off therapy.

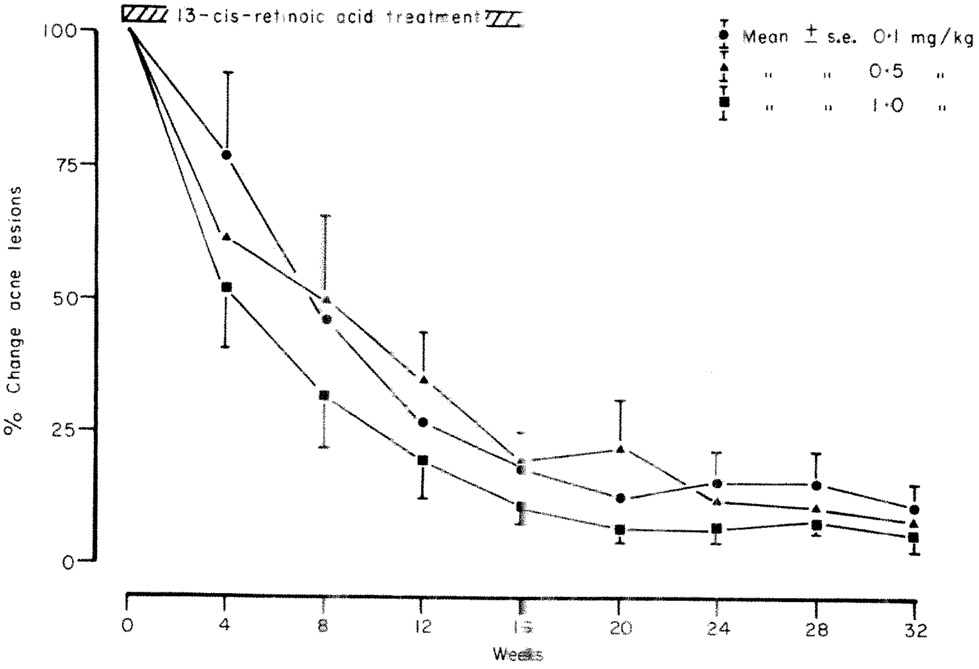

FIGURE 3. Change in acne lesion count during and off therapy.

improvement of greater than 50% in sixty patients over the 16-week treatment period, and a further nine patients continued to improve during the follow-up period (whilst off treatment) to that level. Only seven patients (three in the 0·1, three in the 0·5 and one in the 1·0 mg/kg b.w. dose groups) failed to respond at all. It is interesting to note that both the slow responders and the failures experienced the expected reduction in SER.

Eleven patients (14%) experienced a relapse during the follow-up period, though only six of these to 50% of their pre-treatment acne grade. Five patients were in the 0·1 mg/kg b.w. dose group and three in each of the other dose groups.

Thirteen patients (nine relapsers, four failures) received further treatment prior to the 32 weeks and were withdrawn from the study at that point. Five patients failed to attend during the follow-up period for a variety of reasons.

Side-effects

Clinical. The incidence of clinical side-effects, which were dose related, is as shown in Table 2. Although side-effects were very frequent, the drug schedule had to be altered in only three patients. These three patients were excluded from the study. The severity of the main side-effects is shown in Table 3, and their time course in the 1·0 mg/kg b.w. group is shown in Fig. 4.

TABLE 2. Percentage incidence of clinical side-effects

	Dose group (mg/kg b.w.)		
Side-effects	0·1	0·5	1·0
Cheilitis	90	97	92
Facial dermatitis	48	62	76
Epistaxis	29	31	44
Desquamation	29	28	40
Arthralgia	20	17	28
Conjunctivitis	0	14	20
Malaise	5	7	16

Biochemical. The only abnormalities detected were in the serum γ-glutamyl-o-transferase (SGOT) (Table 4), fasting lipids (Table 5) and urine protein.

The low dose of the drug produced no significant elevation of the mean level of the SGOT. The rises in the other two groups were significant ($P < 0{\cdot}0005$) but only in the 1·0 mg/kg group at the fourth week was the mean rise greater than the upper limit of normal. The normal upper limit for our laboratory is 22 iu/l.

However, thirty-six individuals had raised levels during treatment (four in the 0·1, thirteen in the 0·5 and nineteen in the 1·0 mg/kg group). In only nine cases was this above 30 iu/l, the highest level being 45 iu/l. Only two patients (both in the 1·0 mg/kg group) had consistently

TABLE 3. Severity of side-effects expressed as percentage frequency

	% Mild	% Moderate	% Severe
Cheilitis (mg/kg b.w.)			
0·1	19	57	14
0·5	21	62	14
1·0	20	48	24
Facial dermatitis (mg/kg b.w.)			
0·1	19	29	0
0·5	17	31	14
1·0	24	36	16
Epistaxis (mg/kg b.w.)			
0·1	24	5	0
0·5	31	0	0
1·0	4	36	4
Desquamation (mg/kg b.w.)			
0·1	24	5	0
0·5	14	4	0
1·0	24	16	0

raised levels. There was no consistent pattern in the remaining patients, with nineteen having only a single elevated level.

All levels returned to normal after stopping treatment.

There was no elevation of the mean levels of fasting plasma lipids above the normal range in any of the dose groups. The upper limit of normal for the plasma triglyceride is 1·7 mmol/l and 7·0 mmol/l for the plasma cholesterol. However, there was a steady rise of the plasma triglycerides in all groups during treatment. The values at 16 weeks were significantly different from the baseline levels ($P < 0{\cdot}005$); the levels returned to baseline on stopping therapy. Six patients (three in the 1·0 mg/kg group) had raised levels above 1·7 mmol/l during treatment. The highest level was 2·5 mmol/l, and in three of these patients the levels returned to normal during treatment.

Similar changes were observed in the fasting plasma cholesterol level with no elevation of the mean group level above normal, but there was a steady rise during treatment producing significantly different levels at 16 weeks ($P < 0{\cdot}0025$), and a return to baseline levels after treatment. Eleven individuals had increased levels above normal during treatment, the highest level being 7·4 mmol/l. In six patients, those levels returned to normal during treatment, and the others did so during follow-up. Three patients had increased levels prior to treatment. One suffered no further elevation, and in the other two, levels rose from 8·2 to 9·0 mmol/l and 7·7 to 9·2 mmol/l. These returned to baseline post-treatment.

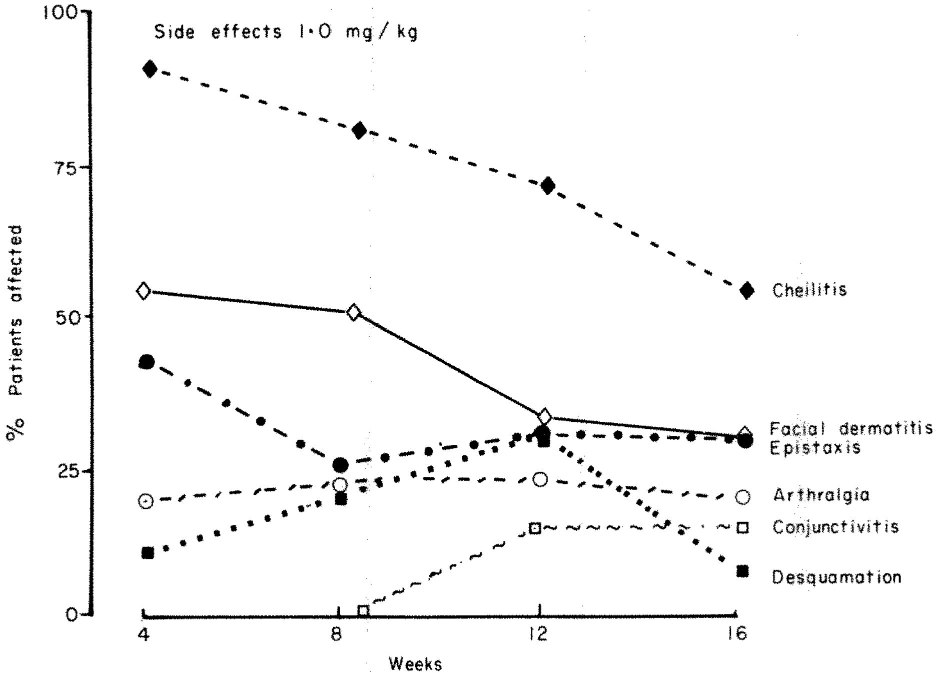

FIGURE 4. Time course of side-effects in patients treated with 1·0 mg/kg b.w. of 13-cis-retinoic acid.

TABLE 4. Serum γ-glutamyl-o-transferase levels [mean, standard error of mean (SE) and number of patients (*n*)]

Dose	Weeks					
(mg/kg b.w.)	0	4	8	12	16	Post
0·1 Mean	19·7	19·2	17·55	18·47	19·47	19·36
SE	1·37	5·21	1·07	0·98	1·84	1·82
n	20	20	20	19	19	11
0·5 Mean	15·1	17·0	18·5	18·9	18·1	18·73
SE	0·63	0·70	0·76	1·27	1·09	0·97
n	27	27	28	25	25	15
1·0 Mean	18·0	25·52	22·2	21·65	21·20	17·17
SE	0·88	1·20	1·56	1·35	0·81	0·77
n	23	21	21	20	20	12

DISCUSSION

This study has confirmed our preliminary observations (Jones *et al.*, 1980; Jones *et al.*, 1981) and those of Farrell *et al.* (1980) and Plewig *et al.* (1980) that this drug is effective in the treatment of acne. Peck *et al.* (1982) has now reported on a double-blind randomized study using a placebo group and also confirms his earlier results.

TABLE 5. Fasting plasma triglyceride levels [mean, standard error of mean (SE) and number of patients (*n*)]

Dose (mg/kg b.w.)		Weeks					
		0	4	8	12	16	Post
0·1	Mean	0·65	0·84	0·80	0·87	0·87	0·67
	SE	0·07	0·10	0·09	0·08	0·10	0·07
	n	21	19	20	18	17	8
0·5	Mean	0·58	0·76	0·79	0·87	0·87	0·80
	SE	0·05	0·07	0·08	0·09	0·10	0·08
	n	29	28	27	28	23	8
1·0	Mean	0·73	0·78	0·93	1·12	1·13	0·74
	SE	0·12	0·08	0·13	0·12	0·13	0·09
	n	21	19	22	20	21	7

The main drive in the production of acne is an increased SER (Cunliffe & Shuster, 1969). It is almost certain that this drug works by reducing sebaceous gland function (as is shown by its ability to reduce the SER) and by altering surface lipid composition (Strauss *et al.*, 1980). This reduction is rapid, reaching almost maximum suppression by 4 weeks. There was a dose relationship in reduction of SER and as all three doses suppressed SER 75–90%, it is likely that smaller doses will have an effect. Current work shows that 0·05 mg/kg b.w. will reduce SER by 50%.

The anti-seborrhoeic effect of 13-cis-retinoic acid is not hormonal. It probably acts directly on the sebaceous gland differentiation (Gomez & Moskowitz, 1980; Gomez, 1981; Sansone-Bazzano, Seeler & Cummings, 1980; Landthaler *et al.*, 1981). The effect of the drug is not permanent, although a reduction in SER is still evident 16 weeks after cessation of therapy, in those patients on the higher dosage. These findings agree with those of Strauss & Stranieri (1982).

The drug has a dramatic effect clinically, particularly when it is realized that most of these patients had unresponsive acne. Ninety-one per cent of patients had a greater than 50% improvement and 66% have not experienced a relapse over the 16-week follow-up. This lack of rapid relapse ran counter to most of these patients' previous histories. Thus, not only is this drug effective clinically, but it can produce a prolonged remission. Though our numbers are small, it appears that patients treated with the low dose have the greater chance of relapse.

The continued improvement after stopping therapy fits with the mechanism of action of the drug. 13-cis-retinoic acid reduces SER and so reduces the drive to the development of new acne lesions. The early reduction in non-inflamed lesions suggests that the drug may have a direct effect on comedogenesis. Current electron microscope work suggests that the drug influences the ultrastructural appearance of the comedones. The fact that most patients experienced a facial dermatitis suggests that the drug also directly affects the epidermis. This, too, may be playing a part in the inhibition of the formation of comedones in the pilosebaceous duct.

This reduction in non-inflamed lesions could subsequently reduce the rate of formation of inflamed lesions. However, it was noted that the numbers of all types of acne lesions were reduced in parallel. The drug may therefore also have a direct anti-inflammatory role (Plewig *et al.*, 1981), or be acting directly against the bacteria which are thought to have a role in this process. This latter suggestion is thought to be unlikely (Weissmann, Wagner & Plewig, 1981), though it was shown in a separate study in more than half our patients that the drug significantly reduced the numbers of *Propionibacterium acnes* on the skin surface. This is likely to be associated with a similar reduction in the follicular colonisation of *P. acnes* and may be secondary to lack of sebum.

However, 13-cis-retinoic acid is not without side-effects (Peck *et al.*, 1979; Jones *et al.*, 1980; Farrell *et al.*, 1980; Wagner & Plewig, 1980; Windhorst & Nigra, 1982). The incidence in this series corresponds to those seen in other centres though the dose relationship has not been emphasized previously. Clinical problems, especially muco-cutaneous ones, are common but only three patients needed to have therapy reduced and these all had a polyarthropathy at 8 weeks and were withdrawn from the study. The side-effects resolved within 1 month of stopping therapy.

The mild and transient abnormalities in urinary protein are probably insignificant and represent mild mucous membrane damage in the urethra.

The assessment of the biochemical abnormalities presents a problem. There seems to be no doubt that the drug causes a rise in the SGOT which presumably represents inflammatory changes in the liver, though no abnormalities of bilirubin or alkaline phosphatase were seen. The effect is dose-dependent with the 1·0 mg/kg dose producing an elevated mean level and having the greatest proportion of patients affected. However, the individual with the highest elevation was taking 0·1 mg/kg b.w. The effects do not persist on discontinuation of therapy. Similar changes occur with the aromatic retinoid, etretinate (Orfanos *et al.*, 1979) though it is not thought to produce a consistent toxic effect (Glazer *et al.*, 1982).

The fasting plasma lipids show a gradual significant rise during therapy but without the mean group levels becoming abnormally high. Certain individuals have shown elevations above the normal limit, half of these being transient and none being particularly high. Those individuals with pre-existing abnormalities showed no marked elevation of their levels by 13-cis-retinoic acid.

The mechanism by which plasma lipids are raised by 13-cis-retinoic acid treatment is not known, though the rise is well reported (Peck *et al.*, 1979; Wagner & Plewig, 1980; Katz, Hildegard Jorgenson & Nigra, 1980; Dicken & Connolly, 1980). It is a feature seen with toxic doses of naturally occurring vitamin A acid (Kligman, Leyden & Mills, 1981) and abnormal lipid handling by the liver is the most likely explanation for this observation (Goodman, 1981; Gerber & Erdman, 1982).

The clinical side-effects are similar to those seen in vitamin A toxicity (Kligman *et al.*, 1981). This suggests that the drug may be working at similar receptor sites to vitamin A or it may displace natural vitamin A in the plasma and allow it to produce the toxic effects. More data on the pharmacokinetics of the drug is required before the precise mechanisms are known.

Long-term treatment with oral antibiotics produces a 27% improvement at 4 months and side-effects in 15% of patients (Cunliffe *et al.*, 1981). These are mainly gastro-intestinal upsets or vaginal candidosis. Abnormalities of routine blood investigations are extremely rare.

Thus, 13-cis-retinoic acid appears to be clinically more effective in severe acne than oral antibiotics, but it is associated with considerably more side-effects.

The choice of an optimum dosage is difficult, requiring a balance of clinical effectiveness, side-effects and relapse rate. The 1·0 mg/kg b.w. dose was associated with the highest incidence of side-effects, especially raised SGOT and fasting lipids. The 0·5 and 0·1 mg/kg b.w. doses were equally effective clinically and these patients encountered far less severe side-effects, but the 0·1 mg/kg b.w. group had far more problems in terms of treatment failures and relapses: 45% *vs* 27–33%. Our recommendation, therefore, would be for the 0·5 mg/kg b.w. dose to be the first choice treatment in antibiotic-resistant cysto-nodular acne.

Further work comparing this dose against long-term oral antibiotics is needed, and is planned for the future.

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Low dose prednisolone or oestrogen in the treatment of women with late onset or persistent acne vulgaris

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SUMMARY

Women with persistent or late onset acne vulgaris were divided into two treatment groups. The first received continuous low dose prednisolone to suppress ACTH-dependent androgen secretion and the second was treated with cyclical oestrogens with medroxyprogesterone to elevate sex hormone binding globulin (SHBG). In both groups there was a significant improvement in the severity of the acne after 4 months' treatment. Although pre-existing hormonal abnormalities were corrected, the improvement in the acne did not correlate with the baseline hormone levels. However, suitable oestrogen–progesterone combinations or low dose prednisolone may be given, alone or in combination, to suppress free circulating androgens in these patients with a high probability of improving their acne.

In an assessment of androgen status in fifty women with persistent or late onset acne vulgaris (Darley *et al.*, 1983) elevated circulating androgens or low SHBG were found in 52% of patients. Raised total serum testosterone (22%) and low SHBG (26%) were the commonest abnormalities, although raised serum dehydroepiandrosterone (DHA) (16%) and DHA sulphate (12%) were also observed. The aim of the present study was to determine whether correction of these abnormalities resulted in clinical improvement in the acne. Low dose prednisolone was used to suppress androgen levels and oestrogens were administered in order to elevate SHBG.

METHODS

Thirty-eight women were investigated. Assay and clinical assessment methods were similar to

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those already described (Darley *et al.*, 1983). Systemic antibiotics were discontinued at least 2 weeks prior to entry into the trials. Patients with high serum testosterone levels were placed in group 1 and patients with normal values of serum testosterone and low SHBG were placed in group 2. Patients with normal testosterone and SHBG results were distributed randomly between the two groups. This work was approved by St Bartholomew's Hospital Ethical Committee.

Patients in group 1 received prednisolone 2·5 mg on waking and 5 mg on retiring. Patients in group 2 received ethinyl oestradiol 30 μg daily and medroxyprogesterone acetate 5 mg on days 1–7 of each calendar month. At the end of this 4-month period, patients attended for clinical (observer) assessment of improvement (Darley *et al.*, 1983). Patients were asked to state whether their acne was slightly better, better, much better, slightly worse, worse or much worse (subjective assessment). While still on treatment all patients had samples taken for androgens and SHBG between 09:00 and 10:00 h.

Non-parametric statistical analysis was chosen because the results were frequently not normally distributed and because the treatment groups were made up of small numbers. For comparison of groups of data, paired rank sum tests were employed. The Spearman correlation coefficient was used for correlations.

Group 1 (Prednisolone)

Fourteen patients received treatment for a total of 4 months. At the end of this period there was a significant improvement in observer assessment ($P < 0{\cdot}01$) (Fig. 1). Three patients felt their acne was better, seven patients were much better and four patients reported no change.

Testosterone, androstenedione, androstenediol, DHA and DHA sulphate were significantly

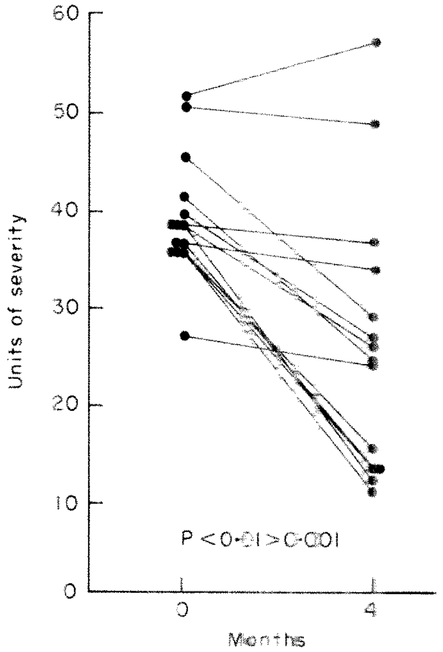


FIGURE 1. Group 1 (Prednisolone). Observer assessment of improvement after 4 months' active treatment in fourteen females with acne vulgaris.

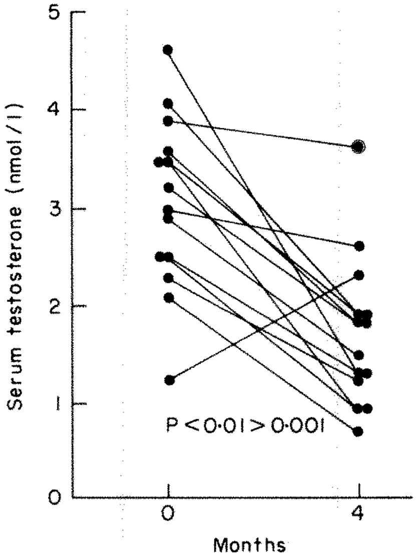


FIGURE 2. Group 1 (Prednisolone). Serum testosterone after four months' active treatment in fourteen females with acne vulgaris. The patient with an unsuppressed cortisol level is circled.

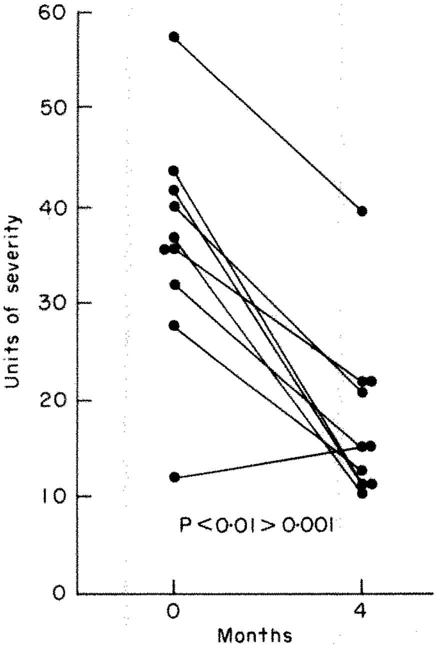
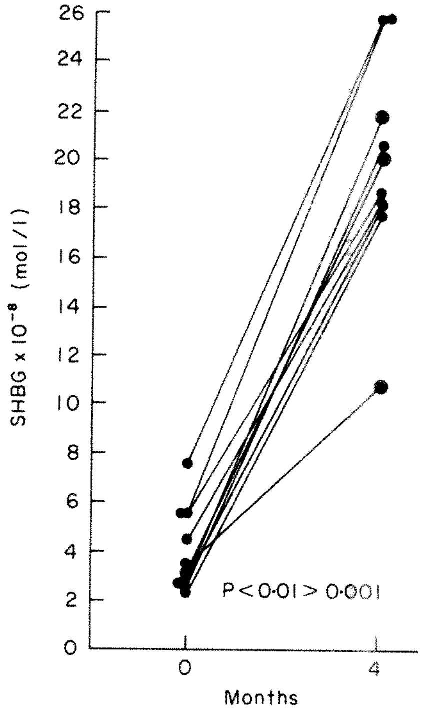


FIGURE 3. Group 2 (Ethinyl oestradiol and medroxyprogesterone acetate). Observer assessment of improvement after four months' active treatment in ten females with acne vulgaris.



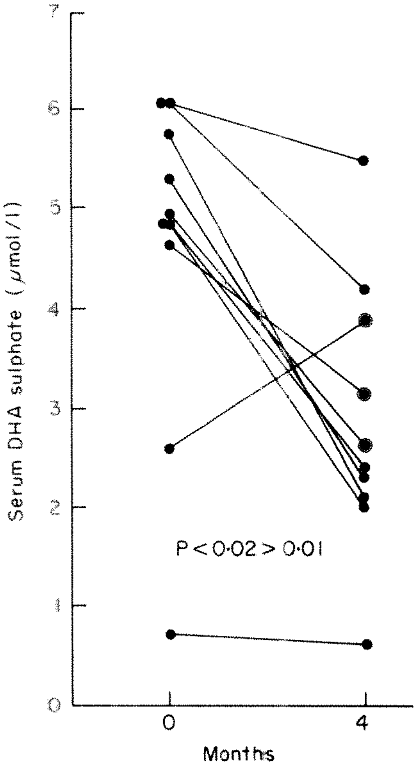


FIGURE 4. (Left) Group 2 (Ethinyl oestradiol and medroxyprogesterone acetate). SHBG after 4 months' active treatment in ten females with acne vulgaris. Patients taking medroxyprogesterone at the time of sampling are circled.

FIGURE 5. (Right) Group 2 (Ethinyl oestradiol and medroxyprogesterone acetate). Serum DHA sulphate after 4 months' active treatment in ten females with acne vulgaris. Patients taking medroxyprogesterone at the time of sampling are circled.

lower in all patients after 4 months' treatment (Fig. 2). Levels were suppressed to within the normal range except in one patient, who had an unsuppressed cortisol level of 670 nmol/l (laboratory normal range 160–700 nmol/l). The androgen level is circled on the graph. SHBG was unchanged after 4 months' treatment.

Four out of eight patients with irregular menses who were treated with prednisolone for 4 months noted that their periods had become more regular.

Group 2. (Ethinyl oestradiol 30 μg daily and medroxyprogesterone acetate 5 mg for 7 days each calendar month)

A total of ten patients completed 4 months' treatment. There was a significant improvement in observer assessment after this period ($P < 0{\cdot}01$) (Fig. 3). Three patients reported that their acne was better and seven patients felt it was much better.

There was a significant increase in SHBG after 4 months' treatment ($P < 0{\cdot}01$) (Fig. 4). Testosterone was significantly higher ($P < 0{\cdot}01$) but androstenedione, androstenediol and DHA

TABLE 1. Adverse side-effects in women with acne who received prednisolone (group 1). Eighteen patients started on treatment

Side-effects	Necessitated stopping: No	Necessitated stopping: Yes
Headaches. Vaginal discharge	1	
Heavier periods	2	
Dysmenorrhoea	1	
Increase in facial hair	1	2
Total	5	2
Other reasons for stopping treatment:		
Failure to improve		1
Inability to tolerate repeated venepuncture		1
Total		2

were not significantly different at the end of the trial. Levels of DHA sulphate were significantly lower after 4 months' treatment ($P < 0.02$) (Fig. 5).

Attempts were made to correlate the changes in severity (i.e. degree of improvement) in group 1 and group 2 with all the baseline values, both clinical and hormonal, but no significant pattern emerged. No serious adverse side-effects were encountered in the course of the clinical trials (Tables 1 & 2). However, treatment was discontinued in eight patients (two in group 1, six in

TABLE 2. Adverse side-effects in women with acne who received ethinyloestradiol and medroxyprogesterone acetate (group 2). Twenty patients started on treatment

Side-effects	Necessitated stopping: No	Necessitated stopping: Yes
Heavy prolonged periods	3	2
Midcycle bleeding	3	1
Breast nodularity		1
Nausea		1
Weight increase	1	
Multiple complaints		1
Total	7	6
Other reasons for stopping treatment:		
Failure to improve		2
Became pregnant		1
Defaulted		1
Total		4

group 2) as a result of adverse side-effects. One patient became pregnant while on ethinyl oestradiol and medroxyprogesterone acetate and the treatment was therefore stopped. A further patient became pregnant within a month of stopping treatment in spite of having experienced difficulty in conception prior to treatment.

DISCUSSION

Continuous low dose steroids have been used for some time as a means of suppressing elevated androgen levels in women with idiopathic hirsuties and the polycystic ovary syndrome (Perloff *et al.*, 1958; Casey *et al.*, 1966; Ettinger *et al.*, 1973). Prednisolone 2·5 mg on waking and 5 mg on retiring (Besser & Edwards, 1972) was used in the present study. This 'reversed circadian rhythm' regimen suppresses ACTH-dependent androgen secretion. The dose used is a replacement dose so the side-effects associated with supra-physiological doses of exogenous corticosteroid administration are not seen.

Prednisolone in this dosage produced significant observer improvement and ten out of fourteen patients reported a definite improvement in their acne. Low dose prednisolone has been used in women with acne with beneficial results by a number of other groups (Didcoct, 1954; Danto, 1957; Goth & Nebenfuhrer, 1964) but androgen levels were not monitored.

All the androgens measured were significantly lower after 4 months' treatment. In one patient suppression was minimal and was less than that seen after dexamethasone. A high cortisol level at 4 months suggests that this individual was not taking prednisolone at the time of sampling. Apart from this instance all androgens were suppressed to within the normal range after 4 months' prednisolone.

In hirsuties suppression of androgens occurs in the majority of patients but the clinical response is variable and is unrelated to pre-treatment levels (Ettinger *et al.*, 1973). Ettinger *et al.*, (1973) also noted that androgen levels may be permanently restored to normal by continuous low dose steroids.

Eighteen patients were started on this treatment. Three patients reported an increase in facial hair while on prednisolone although this was not clinically apparent. In one patient this complaint was temporary and prednisolone was continued but in the other two, treatment was stopped. One of these patients complained of hirsuties after only 10 days of therapy. Other side-effects were encountered in four patients and were temporary and not troublesome.

Four out of eight patients who had irregular periods at presentation reported an improvement in the regularity of their cycles after 4 months' prednisolone. This is a frequent finding in women with hirsuties and the polycystic ovary syndrome treated with this regime (Besser & Edwards, 1972; Ettinger *et al.*, 1973). Patients should be warned that they may become more fertile.

The second group of patients were treated with ethinyl oestradiol 30 μg daily and medroxyprogesterone acetate 5 mg for 7 days each month. It was chosen because the combination produces a marked increase in SHBG using a low dose of ethinyl oestradiol. The progestogen is essential to ensure regular stripping of the endometrium and prevent endometrial hyperplasia.

Observer assessment demonstrated a significant improvement after 4 months' treatment. All ten patients who completed treatment felt that there had been an improvement in their acne. The beneficial effect of oestrogens has been a consistent finding in acne. Palitz (1962) was the first to use a combined oestrogen–progestogen contraceptive pill in acne. It has been pointed out that improvement in acne may not occur for three or four cycles and there may be a slight deterioration in the first few weeks of therapy (Strauss & Pochi, 1963). Clinical trials should therefore be continued for a minimum of 2 months.

The elevation of SHBG produced by ethinyl oestradiol and medroxyprogesterone was impressive. Levels rose by fourfold after 4 months' treatment. Testosterone levels were significantly higher after 4 months of ethinyl oestradiol and medroxyprogesterone. This has not been previously reported but the situation is similar to that seen in pregnancy when SHBG rises and there is an increase in total testosterone. DHA sulphate levels were significantly lower on ethinyl oestradiol and medroxyprogesterone after 4 months. Low levels have been reported before in women on combined contraceptive pills (Bulbrook *et al.*, 1973).

Twenty patients were started on ethinyl oestradiol and medroxyprogesterone. One patient became pregnant on the treatment and a further patient became pregnant during the first cycle after stopping. This combination is not contraceptive and patients should be warned of this. Heavy prolonged periods and inter-menstrual bleeding were the most troublesome side-effects and were encountered in nine patients. These tend to settle if treatment is continued but in three patients it was necessary to stop treatment. In all, six out of twenty patients stopped therapy as a result of adverse side-effects.

The progestogenic 19 nortestosterone derivatives, norethindrone, norgestrel and norethisterone, lower SHBG when given alone. Lynestrenol has a similar but less marked effect. In combination with ethinyl oestradiol or mestranol, the oestrogen-induced rise in SHBG is partially or totally suppressed by these progestogens (Thijssen & Van Kammen, 1975; Victor, Weiner & Johansson, 1976; El Makhzangy, Wynn & Lawrence, 1979). Ethynodiol diacetate and megestrol acetate do not have this effect and preparations containing ethinyl oestradiol and these progestogens stimulate SHBG production (Thijssen & Van Kammen, 1975; Victor *et al.*, 1976; El Makhzangy *et al.*, 1979).

Testosterone is higher in patients taking combined pills which contain norgestrel or norethisterone (Briggs & Briggs, 1972; Kjeld, Puah & Joplin, 1976) while those containing lynestrenol lower testosterone (Browning & Anderson, 1977). Preparations containing ethynodiol diacetate or chlormadinone produce no change in testosterone levels (Weisz *et al.*, 1973).

Preparations containing norgestrel, norethindrone and norethisterone tend to make acne worse while those containing chlormadinone, ethynodiol diacetate and megestrol acetate are beneficial (Barranco, 1974). Ethinyl oestradiol (50 μg) with a lower dose of norethisterone (1 mg) is also helpful in acne (Pye *et al.*, 1977). Apart from their effect on serum testosterone and SHBG, preparations containing 19 nortestosterone derivatives have been shown to stimulate sebaceous glands (Strauss & Kligman, 1961).

As far as the effect of the combined pill on acne and sebum excretion rate is concerned it is clear that the changes in SHBG and testosterone are dependent on the dose of oestrogen and the dose and type of progestogen used. Preparations which contain ethinyl oestradiol and ethynodiol diacetate increase SHBG levels without altering serum testosterone. Levels of circulating free testosterone will therefore fall. These preparations have been beneficial in the treatment of acne in uncontrolled trials in our hands. The most effective way to lower free testosterone levels is to combine low dose prednisolone with a suitable combined pill and this regime has been used in the treatment of hirsuties (Anderson, 1974; Casey, 1975). Saihan & Burton (1980) have used such a combination in a small group of women with acne with beneficial results.

The improvement in the acne in patients treated with ethinyl oestradiol and medroxyprogesterone or with low dose prednisolone did not correlate with the baseline androgen or SHBG results. It is therefore not possible to predict which patients will benefit from this form of therapy. However, certain guidelines for investigation and treatment can be suggested. It may be helpful to measure total serum testosterone and SHBG in women over 18 years old who have troublesome acne. A suitable combined contraceptive pill containing ethynodiol diacetate should be prescribed in women who are found to have low SHBG levels or in any woman suffering from acne who wishes to use this form of contraception. Patients with elevated serum testosterone levels should receive low dose prednisolone. The serum testosterone should be repeated after 1 month to ensure that suppression has occurred. In the most therapeutically resistant patients and in those women with both abnormal testosterone and SHBG levels, the combination of an oestrogen-dominant contraceptive pill and low dose prednisolone will have the most marked effect in lowering free testosterone levels. Assuming a satisfactory response is obtained, therapy should be continued for a year before any attempt is made to withdraw medication.

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Case Reports

Familial sea-blue histiocytosis with cutaneous involvement. A case report with ultrastructural findings

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SUMMARY

A 25-year-old man presented with the sea-blue histiocyte syndrome. In addition to hepatosplenomegaly, lung infiltrate and bone marrow involvement, he had cutaneous nodules and plaques over his face, and thickened eyelids. Typical sea-blue histiocytes were found in the skin biopsy. Electron microscopy showed cytoplasmic bodies revealing a peculiar pleomorphic structure. A sister suffered from the same disease. This is the first familial case of sea-blue histiocyte syndrome showing cutaneous involvement.

Sea-blue histiocytes (SBH) are large macrophages whose cytoplasm is filled with granules staining blue or blue-green with Giemsa. These cells were first observed in the spleen (Moeschlin, 1947). It is now known that they occur primarily in patients with an inherited disorder called the sea-blue histiocyte syndrome (SBHS) (Silverstein, Ellefson & Ahern, 1970). This disease is characterized by an infiltration of SBH in the bone marrow, spleen, liver, lymph nodes, lungs and, less frequently, the eye, nervous system and gut (Sawitsky, Rosner & Chodsky, 1972; Silverstein & Ellefson, 1972). Cutaneous changes which have been reported include facial macular hyperpigmentation (Malinin, 1961; Saidi *et al.*, 1970) and leucoderma (Bednar, Hermansky & Lojda, 1964), but in these cases histological examination of the skin has failed to reveal the presence of SBH.

However, the SBH cells have been reported to occur in other organs such as the bone marrow or spleen in various unrelated diseases, including thalassemia (Beltrami, Bearzi & Fabris, 1973), and hyperlipaemia (Rywlin *et al.*, 1971; Robinowitz *et al.*, 1975). As far as we know, SBH have been reported in the skin in only two patients, one with mycosis fungoides (Robinowitz, Roenigk & Smith, 1975) and one with chronic granulocytic leukaemia (Dosik, Rosne & Sawitsky, 1972). The substance stored in the granules has not been fully identified, but histochemical reactions and histological staining features suggest it is a glyco-phospholipid,

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probably a ceroid (Silverstein & Ellefson, 1972; Reidbord, 1972). Ultrastructural examination shows that the bodies are usually composed of multilamellar membranes arranged in whorls with 5 nm periodicity (Lynn & Terry, 1964). Nevertheless, recent studies have emphasized the pleomorphism of the stored material in SBH from different diseases (Amazon & Rywlin, 1980; Beltrami *et al.*, 1973) and in different cases of the same disease (Kattlove *et al.*, 1970).

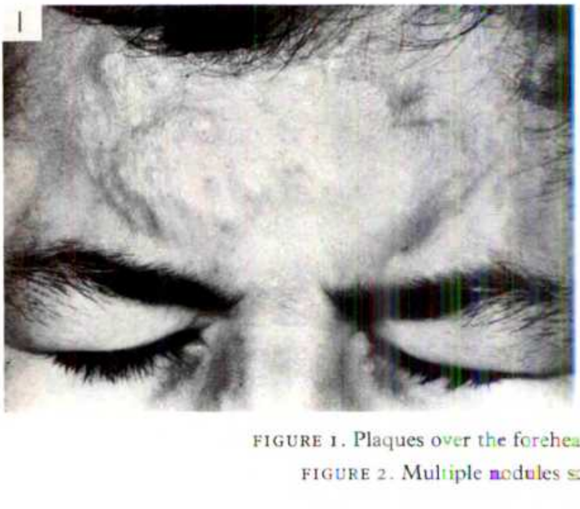
We here describe a case of familial primary SBHS in a man with cutaneous nodules. Electron microscopy of SBH in the skin showed peculiar inclusions never previously reported.

CASE REPORTS

Case 1

G.S., a 25-year-old man, had a long and complex history which has been summarized by Minetto *et al.* (1978). He had hepatosplenomegaly, bone marrow involvement, slight peripheral thrombocytopenia and diffuse pulmonary micronodular involvement. In 1977 the cutaneous lesions were limited to the eyelids. These were described by the authors as 'sclerodermoid' but no skin biopsy was performed. Five years later the patient still felt well despite his persistent disease. He consulted us because nodules had developed on his face. Clinical examination showed multiple nodules and plaques located on the forehead and chin (Figs 1 & 2); they were slightly erythematous with a waxy consistency. The palpebral infiltration had remained unchanged. A skin biopsy revealed a multifocal histiocytic nodular infiltrate located in the middle and the deep dermis, mainly in a perivascular or periadnexal pattern (Fig. 3). There were also a few sarcoid-like granulomas showing epithelioid histiocytes mixed with giant cells and lymphocytes (Fig. 3). The stroma showed a fibrous reaction. Histochemistry showed the cells were typical SBH, since they were filled with granules staining blue-green with Giemsa (Fig. 4), PAS positive, Sudan Black positive and showing a deep blue colour without metachromasia with toluidine blue.

To exclude sarcoidosis further investigations were carried out. A Kveim test was negative; ACE (angiotensin-converting enzyme) was normal, 128 U/ml (NR 50–150). Some leukocyte enzymes known to be deficient in some lysosomal storage disease were studied (the units are nmol/h/g protein): arylsulphatase pH 5, 160 (NR 42–189); pH 6, 105 (NR 25–151);



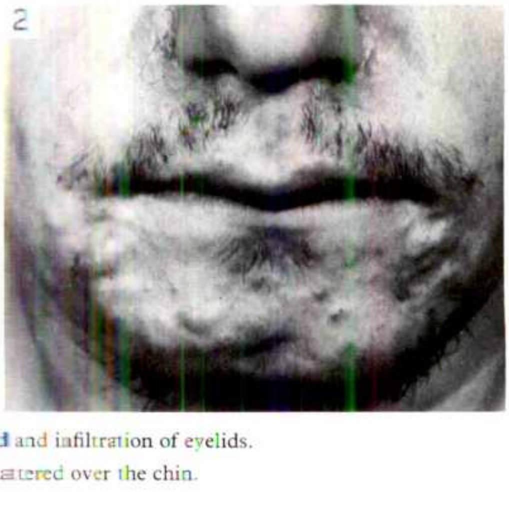


FIGURE 1. Plaques over the forehead and infiltration of eyelids.

FIGURE 2. Multiple nodules scattered over the chin.

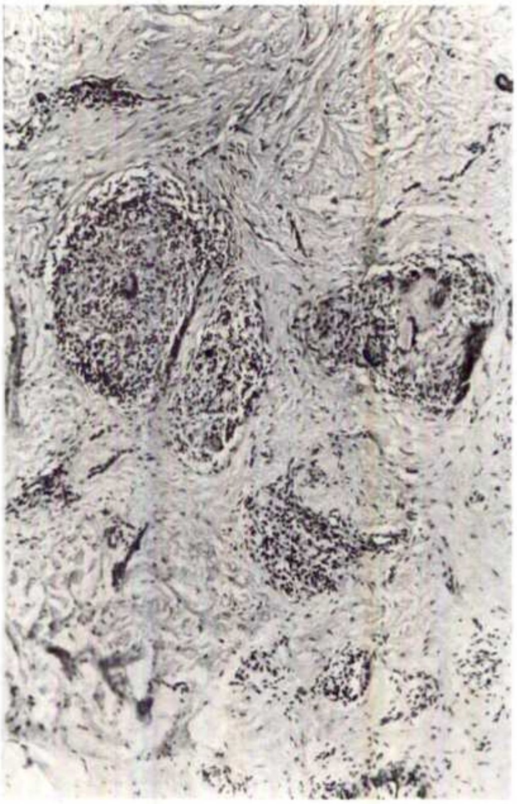


FIGURE 3. Sparse nodules of infiltrate in a fibrous stroma; note the granulomas with giant cells (H & E, × 50).

β-galactosidase 88 (NR 39–193); α-galactosidase 48 (NR 25–106); β-glucosidase pH 4, 2·2 (NR 0·3–4·5); pH 5·3 4·0 (NR 2·8–15·2); α-fucosidase 73 (NR 20–110); α-mannosidase 242 (NR 120–240). Acid phosphatase was normal. Sphingomyelinase could not be studied. Urinary mucopolysaccharide excretion was also normal (uronic acid 3·5 mg/day). Blood tests gave normal results except for platelets (100,000/mm^3) and hypergammaglobulinaemia of 24·2% (total protein 7 g/100 ml; albumin 50·4%; α_1-globulins 3·2%; α_2-globulins 8·5%; β-globulins 13·7%). Pulmonary function was normal. In conclusion, the disease showed a mild course with no progression apart from the skin lesions.

Case 2

A.S., the 17-year-old sister of the first patient, had a history of hepatosplenomegaly and pulmonary infiltrates. In 1978 she was reported as a case of SBHS (Nicola *et al.*, 1978) and the authors were unaware that her brother suffered from the same disease. Histologically SBH were found in muscles and subcutaneous tissue. Nicola *et al.* (1978) reported that the girl had a pale face with diffuse infiltration and thickening of her skin but no dermatological opinion was sought. We have since personally observed the patient and found no skin lesions similar to her brother's, but her skin was slightly indurated with loss of elasticity. These changes were

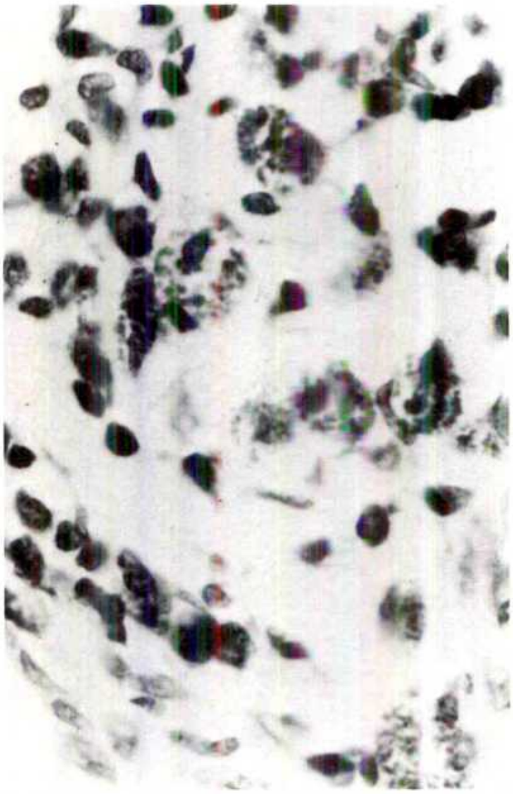


FIGURE 4. Histiocytes showing the cytoplasm filled with blue stained granules (May–Grünwald Giemsa, × 400).

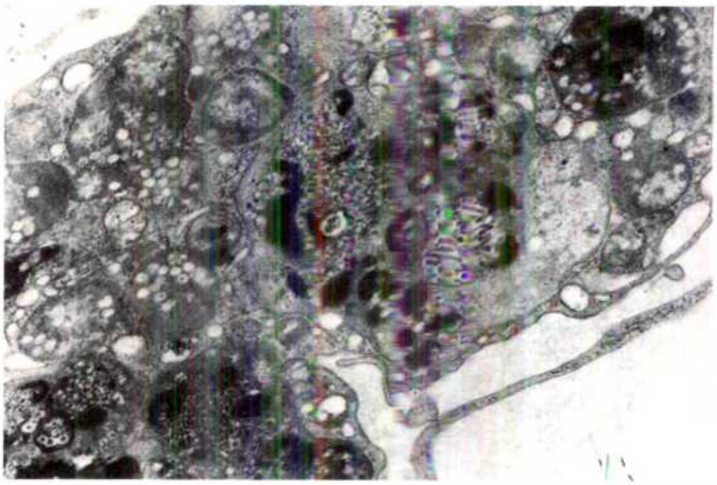


FIGURE 5. The cytoplasm of a sea-blue histiocyte filled with membrane-bound inclusions (× 16,000).

minimal and could be easily missed by an observer ignorant of her disease. She refused a biopsy and further investigation.

Electron microscopy

A nodular lesion on the face of G.S. was biopsied and specimens for electron microscopy were fixed in 3% glutaraldehyde and post-fixed in 1% osmium tetroxide in phosphate buffer to which sucrose had been added. After dehydration in graded alcohols the specimen was embedded in Araldite. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a Siemens 102 electron microscope.

Electron microscopy showed the cytoplasm of the SBH was filled with membrane-bound bodies (Fig. 5). Several types of inclusions were recognizable:

(1) Type 1 were oval or round showing a dense amorphous matrix with areas of different density and small, round electron-lucent spaces (Fig. 6a).
(2) Type 2 were pleomorphic large phagolysosomes showing whorls of lamellated and membranous structures and microfilaments mixed with dense osmiophilic round, polycyclic or irregular inclusions with a finely granular background (Fig. 6b).
(3) Type 3 bodies were characterized by the presence of dense rod-like formations showing a transverse round section varying between 40 nm and 60 nm; their length was 0·2–0·6 μm (Fig. 6c).

All SBH had type 2 bodies, while types 1 and 3 were present in only some cells. Under high magnification the dense osmiophilic material appeared to be amorphous with no discernible periodic pattern. Only after a careful search could we find some bodies showing a fingerprint structure organized as membranes showing a 4·5–5·0 nm periodicity. The osmiophilic lines were about 2·5 nm wide and the clear space delineated by them was 2·0–2·5 nm. No picture suggesting erythrophagocytosis or ingestion of extracellular debris was found. There were also

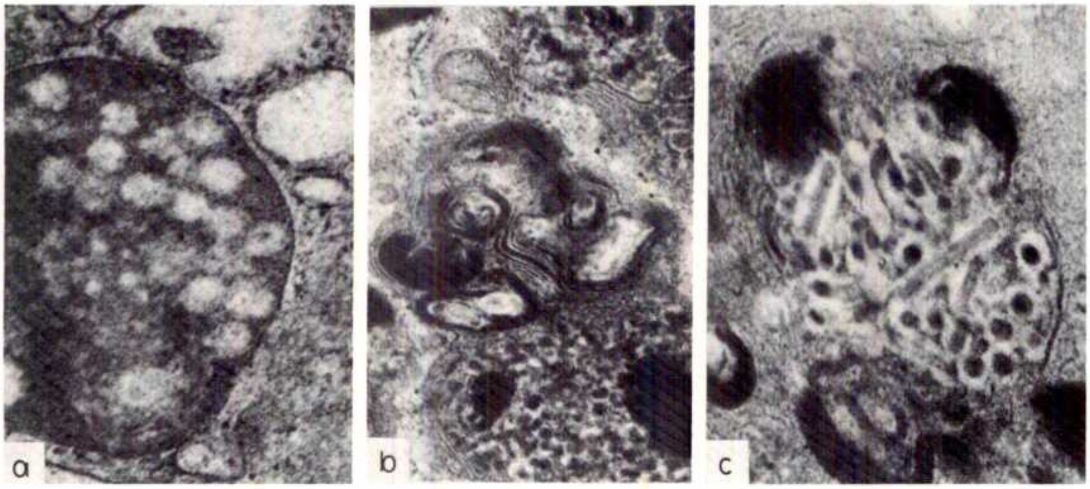


FIGURE 6. Type 1 bodies, showing an amorphous matrix with small electron-lucent areas (× 80,000). (b) Type 2 phagosomes containing filaments, lamellated formations, and dense osmiophilic inclusions emerging from a granular background (× 40,000). (c) Type 3 bodies, showing rod-like formations (× 50,000).

highly vacuolated cells sometimes containing whorls of a membranous substance. These cells had no phagosomes or primary lysosomes while the endoplasmic reticulum was well developed and thus they were identified as fibroblasts.

DISCUSSION

The unusual clinical feature in this case was the presence of nodular cutaneous involvement with infiltrates of SBH; this has not previously been described. Furthermore, electron microscopy revealed the storage material to be different from that observed in other known lipid storage diseases (Volk, Adachi & Schneck, 1972; O'Brien *et al.*, 1975; Martin & Ceuterick, 1978), although it was quite similar to a ceroid substance (Reidbord, 1972). It is generally acknowledged that lipid material together with metabolic debris can be phagocytosed by macrophages, and the stored substance undergoes secondary chemical modifications, resulting in ceroid-like deposits due to slow peroxidative processes. At the end of the process these cells show the staining properties of SBH and thus it is easy to understand why secondary SBH can be found in so many different conditions. Ultrastructurally the material shows a common pattern, with osmiophilic deposits arranged in lamellated and membranous formations frequently showing an internal periodicity of about 5 nm. A certain heterogeneity of the stored material in SBH from different diseases and different patients has been reported by several authors (Amazon & Rywlin, 1980; Kattlove *et al.*, 1970). Such heterogeneity may account for the ultrastructural appearance of the stored material in this case. A striking feature was the presence of the rod-like bodies, although their significance and origin remain obscure. While the occurrence of secondary SBH is explicable, the primary form is still imperfectly understood. It is speculated that the stored material in these cases is a consequence of an enzymatic deficiency which leads to imperfect metabolism of lipids in cells, thus resulting in the storage of a ceroid substance. Since the metabolic disorder has not yet been identified it is possible that the SBHS is not a unique clinical disease and that heterogeneous enzyme defects may result in the same clinical picture. In this family for example, the defect produced the cutaneous deposit of the stored material, a feature not observed in other cases of SBHS. Only further observations of similar cases will show whether we are dealing with a new variant of the syndrome.

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Nail staining due to hydroquinone skin-lightening creams

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SUMMARY

Staining of the nails due to hydroquinone-containing creams is not a well-recognized condition. We report two cases in older women in whom brown discoloration developed after the use of cosmetic skin-lightening creams for actinic lentigines of the hands. The cause of the discoloration and its relation to light exposure is discussed.

Nail discoloration after the use of hydroquinone does not appear to have been reported in the British literature and a relationship to light exposure has not been previously noted.

CASE REPORTS

Case 1

A 69-year-old woman was seen in August 1981 with a 3-month history of brown discoloration of the finger-nails, which started during the fifth week of a sunny visit to Israel. She had been applying skin-lightening creams twice daily to the backs of the hands for actinic lentigines over a period of 8 weeks prior to the development of the nail changes. The creams used were Esoterica (Mitchum–Thayer Ltd) and Fade-Out (Coparel Ltd).

Examination revealed pale brown discoloration over the distal and lateral aspects of all the finger-nails (Fig. 1). On stopping the skin-lightening creams the nail changes resolved after 2 months.

Case 2

A 57-year-old woman was seen in November 1981 with a complaint of brown discoloration of the finger-nails. This began in March 1980, resolved in October 1980 and recurred during March 1981. When seen she felt that the brown colour was fading again. Over the previous 3 years she had applied Fade-Out cream or Fortified Esoterica cream (Mitchum–Thayer Ltd) twice daily to the backs of the hands and forearms for actinic lentigines. She noted that the Fortified Esoterica cream darkened with age.

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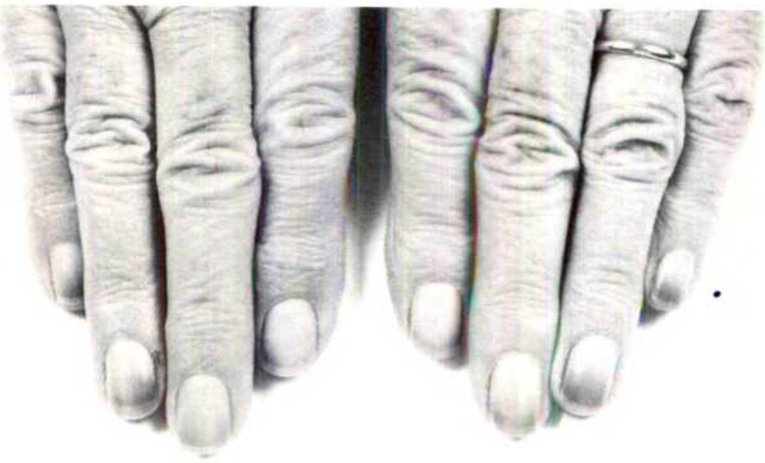

FIGURE 1. The appearance of the nails.

Examination showed brown discoloration of all the finger-nails. On stopping the creams the nail changes almost completely resolved after 1 month.

DISCUSSION

Nail discoloration after the use of hydroquinone does not appear to have been reported in the British literature, and its relationship to light exposure has not been previously noted. However, Arndt & Fitzpatrick (1965) reported three patients in the United States with discoloured finger-nails following the use of 2% and 5% hydroquinone-containing creams in the treatment of vitiligo, chloasma, freckles and post-inflammatory hyperpigmentation. Also Garcia, White & Willis (1978) in the United States noted diffuse orange-brown nail staining after the use of Esoterica cream by several patients. Garcia *et al.* (1978) considered that the colour change was due to the oxidation products of hydroquinone and that a similar colour change could be seen on the surface of the cream after exposure to air.

Hydroquinone is readily oxidized to quinone, a yellow compound, and quinone subsequently undergoes oxidation to hydroxyquinone. The oxidation of quinone is a photosensitive reaction. Hydroxyquinone, another yellow compound, is unstable and polymerizes to products which are dark brown, and this reaction is also photosensitive (Sterner, Oglesby & Anderson, 1947). These oxidation and polymerization products are also probably the cause of the brown pigmentation which affects the cornea and conjunctiva of workers involved in the manufacture of hydroquinone, in which quinone is an intermediate (Sterner *et al.*, 1947). The spontaneous resolution of the nail staining during the winter months in our second case suggests that light is playing a role in the production of the staining. Light may be involved in the eye changes due to hydroquinone/quinone (Anderson, 1947), and is implicated in the development of ochronosis and pigmented colloid milium of the facial skin in Negro patients due to hydroquinone bleaching creams (Findlay & de Beer, 1980).

Both our patients applied the creams to the backs of the hands and we believe that on applying the creams significant amounts became directly incorporated into the nail plates. Garcia *et al.* (1978) and Arndt & Fitzpatrick (1969) do not state where their patients with nail discoloration had been applying the creams. The lack of reported nail involvement in Negro patients using such creams may be because the creams were applied to the face rather than to the backs of the hands, or because any induced pigmentation is masked by the normal pigmentation of Negro skin, or there could be unconcern about any such nail changes.

Hydroquinone-containing creams are easily obtainable 'over the counter' and the following creams were found to be available in the Bristol area: Nudit Gentle Skin Lightener (Helena Rubenstein), Fade-Out (Coparel Ltd), Artra (Plough Inc.), Ambi Special (Nicholas Lab. Inc.), Nadinola (Chattem Labs.), Esoterica (Mitchum–Thayer Ltd.), Fortified Esoterica (Mitchum–Thayer Ltd.), and Palmers Skin Success Cream (E.T. Browne Drug Co.). The maximum permitted concentration of hydroquinone in such products in Common Market countries is 2% (Council Directive, 1976).

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Pigment streaks in the nail plate due to secondary malignant melanoma

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SUMMARY

We describe two patients with pigment streaks in the nail plate due to secondary malignant melanoma in the nail matrix.

It is well known that a primary malignant melanoma may develop in the nail matrix and produce a pigment streak in the nail plate. A similar streak may be caused by a benign junctional naevus in the nail matrix (Noble, Ferrin & Merandino, 1952). Less well-defined streaks are very common in coloured persons and are due to the presence of dopa-positive melanocytes without amounting to a true naevus (Leyden & Spott, 1972). It is much less well known that secondary deposits of malignant melanoma in the nail matrix may produce deeply pigmented bands in the nail plate. One such report is given by Kolmsee & Schultka (1972) with photographic evidence of a 60-year-old man who had a primary melanoma below the right nipple of 3 years duration and with widespread metastases. Also a report of secondary malignant melanoma of the nail bed is given by Zaun (1980) in his book. We now report two patients with a similar metastatic manifestation of their disease.

CASE REPORTS

Case 1

A 27-year-old male presented in December 1976 with a primary malignant melanoma on the right pectoral region, for which he had a wide excision and a skin graft. The primary tumour measured 6 mm in thickness and invaded the subcutaneous fat (Clark's level V). The first blood-borne cutaneous metastasis confirmed histologically appeared in June 1977. By December 1977 widespread dissemination had occurred and despite chemotherapy, the disease continued to advance. In April 1978 a pigmented band appeared on the nail plate of the left big toe (Fig. 1). Pigmented streaks were also noted on the nail plates of two fingers of the right hand

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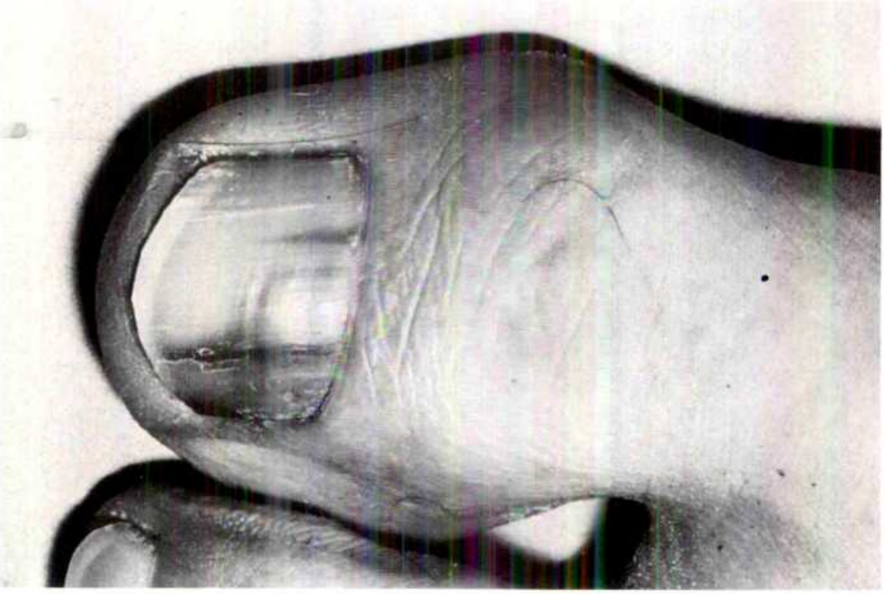

FIGURE 1. Pigmented band on the nail plate of the left big toe in case 1.

and one finger of the left. By July 1978 streaks were more marked and deeply pigmented and involved all the nail plates of both hands (Figs 2 and 3). The patient died in October 1978.

Post-mortem examination confirmed extensive metastatic disease involving practically every organ including the brain and myocardium.

Case 2

A 58-year-old female presented in February 1977 with a primary malignant melanoma on the inner quadrant of the left breast which was widely excised. The primary tumour measured 2·8 mm in thickness and infiltrated to Clark's level III. In August 1977 she had a left axillary block dissection for histologically involved lymph nodes by malignant melanoma. She commenced BCG immunotherapy in September 1977 and remained in apparent remission on this treatment until May 1981, when subcutaneous metastases appeared and were histologically confirmed. In June 1981 cerebral metastases were diagnosed on a CAT scan. At the same time pigmented streaks were noted on the plates of two fingers on the right hand (Fig. 4). The patient died from a rapidly expanding cerebral tumour early in July 1981.

DISCUSSION

The appearance of pigmented streaks in the nail plates of these two patients is an unusual metastatic manifestation of malignant melanoma. Although histological confirmation has not been obtained, the widespread dissemination of the disease in both cases is testimony to their nature. Furthermore, the proliferation and 'growth' of these streaks in the first patient is also characteristic of their metastatic nature.

In a series of 230 patients with metastatic malignant melanoma seen in the Medical Oncology Unit at Westminster Hospital in the last 5 years, such lesions have been recorded only in the two

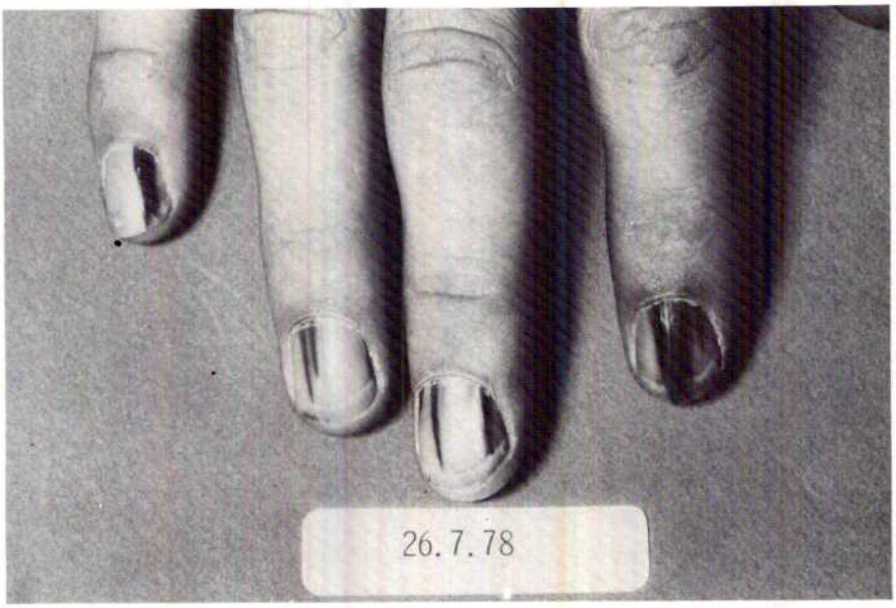

FIGURE 2. Multiple pigmented streaks on nail plates. Case 1.

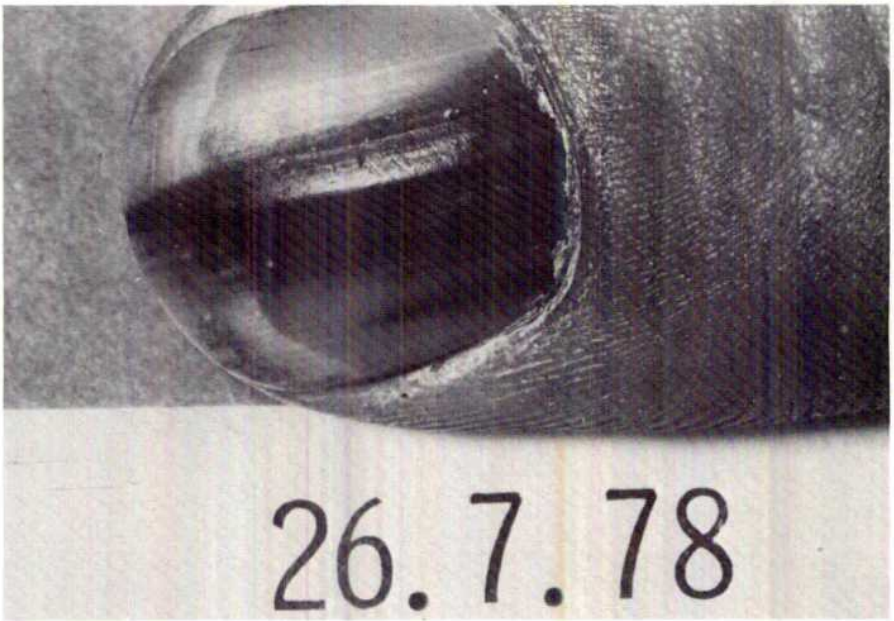

FIGURE 3. Close-up view of the lesion on the nail plate of right index finger. Case 1.

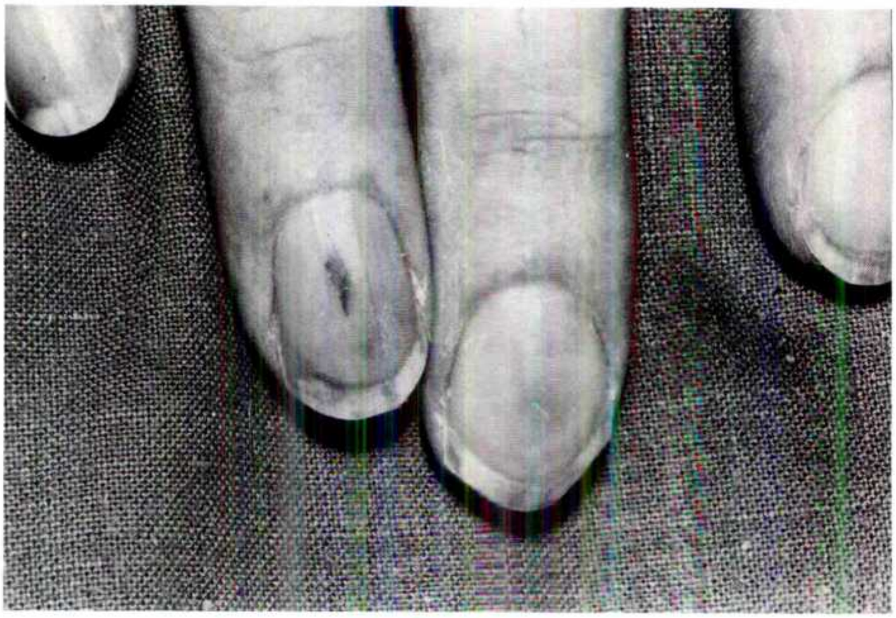

FIGURE 4. The appearance of the lesions in case 2.

patients of this report (less than 1%). In our experience the presence of such lesions in a case of malignant melanoma is an indication of widespread melanomatosis.

ACKNOWLEDGMENTS

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Comment

DRUGS THAT ARE INEFFECTIVE IN THE TREATMENT OF ACNE VULGARIS

A conspicuous feature of the literature on the therapy of acne is the number and diversity of substances which have been found to be beneficial in therapeutic trials (Cunliffe & Cotterill, 1975; Frank, 1971). Few of these ever prove worthwhile in the hands of anyone but the authors. This extraordinary outcome is usually attributed to the placebo effect which seems to exert an unusually powerful influence in this disease (Fry & Ransey, 1966; Lowinger & Dobie, 1969). Alternative explanations for the consistently beneficial effects of exotic, non-rational, curious substances are too ill-mannered to be recorded.

Drug testing is a very difficult enterprise, especially for chronic diseases which profoundly affect the quality of life of the sufferers. All parties, the patient, doctor and parents are hopeful of a quick victory. Negative results arouse anxiety and hostility. Denial of ineffectiveness is a common psychological device to help sustain hope in the face of failure. It is also noteworthy that placebos are far more effective in certain hands and places than others. This is partly based on social and geographical influences. Doctors located in upbeat, sunny prosperous communities where 'happiness' is obligatory are particularly likely to have high efficacy ratings. Cold, foggy, moody climates reduce the potency of placebos and probably of rational drugs also.

We now wish to declare that the Philadelphia area is apparently an unfavourable place for evaluating acne therapies. The placebo effect is especially feeble in the environment of the University of Pennsylvania. In our habitat the disease seems to be sullen and stubborn, showing less drastic fluctuations in intensity from one week to the next. The valiant patients whom we have persuaded to forego treatment for 8 weeks under our close surveillance have mostly presented the same clinical picture at the end as at the start. The disease has not been swerved in its course over a 2- to 3-month period despite manoeuvres which might be powerful in other places, viz., daily facial massage by professionals (laying on of hands), cold compresses (freeze out the scourge) and steamy saunas (volatilization of bad humours). We have an extensive record of pessimistic patients who refused to be improved by these exhibitions.

We have been searching for more effective drugs for about two decades. Our screening programme is as follows. After preliminary evaluation of toxicity, topical agents are applied twice daily for 8 weeks to ten patients with moderately severe acne using the highest concentration (not exceeding 10%) that will not be excessively irritating. The dose of drugs to be used orally is dependent on what is known of their pharmacological and toxicological effects in humans. The patients are seen every 2–3 weeks. The judgement of benefit is purely clinical, by global evaluation. Scores of pilot screenings over the years have created a context of experience with which to compare a result. Drugs which reduce disease severity by less than 25% are considered ineffective. We are interested only in agents which have an unequivocal impact on the disease in relation to treatments generally regarded as efficacious. We have the conceit to believe that global ratings by experienced observers are more credible than the

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scientific style of using lesion counts and suave statistics. In fact, we think there is often a direct correlation between mendacious work and the statistical support system.

The purpose of this communication is to provide a list of drugs which do not work in acne. These are as follows:

TOPICAL AGENTS

Acids

5·0% formic acid (aqueous)
10% glycolic acid (aqueous)
1·5% iodoacetic acid in ethanolic solution
2% monochloracetic acid in water and propylene glycol (90:10)
5·0% peracetic acid (aqueous)
5·0% periodic acid (aqueous)
5% phenol (aqueous)
7·5% p-iodophenol (ethanolic solution)
10% pyruvic acid (aqueous)
2·0% trichloracetic acid (aqueous)

Surfactants and soaps

10% Ajax® in aqueous suspension (household cleansing powder)
2·0% benzalkonium chloride (aqueous)
2·0% cetylpyridinium chloride (aqueous)
50% Ivory® liquid detergent (aqueous)
5% sodium lauryl sulfate (aqueous)
5% toilet soaps (aqueous)

Antimicrobials

5·0% chlorhexidine in ethanol:water (1:1)
chlorine dioxide (aqueous) (1:1)
2·0% chloroxine in dimethylacetamide
2·0% chloroxine (ethanolic)
2·0% 8-hydroxyquinoline in ethanol:propylene glycol (85:15)
2·0% hexachlorophene (alcoholic gel)
5·0% iodochlorhydroxyquin (vanishing cream)
2·0% isoniazid (aqueous)
2·0% p-chloro-xylenol (ethanolic)
5·0% pentachlorophenol (ethanolic)
selenium sulfide [Selsen Suspension, Abbott]
3·0% 6-chloro-2-phenylphenol (ethanolic) (85:15)
2·0% sodium omadine (aqueous)
5·0% tetrachlorophenol (ethanolic)
2·0% tribomosalicylanilide (alcoholic gel)
2·0% trichlorocarbanilide (alcoholic gel)
5% zinc peroxide (vanishing cream)
2·0% zinc pyrithione (alcoholic gel)

Antibiotics

2·0% chloramphenicol (ethanolic)
2·0% kanamycin (aqueous)
2% minocycline hydrochloride (vanishing cream)
2% neomycin sulfate (aqueous)
2·0% penicillin G (aqueous)
2·0% rifampin in dimethyl sulfoxide (DMSO)
2% tetracycline (alcoholic)
2% tetracycline base in dimethylacetamide
2% tetracycline hydrochloride (ethanolic)
2·0% tobramycin (aqueous)

Miscellaneous

acetone (5 times daily)
5% acetylcysteine (ethanolic)
5% acetylsalicylic acid (ethanolic)
10% aluminum chloride (aqueous)
0·3% colchicine in dimethylacetamide
10% dicholoronaphthoquinone in dimethylacetamide
dimethylformamide
25% dimethyl sulfoxide (DMSO)
1·0% 5-fluorouracil (Fluoroplex®, Herbert)
5·0% resorcinol (lotion)
10·0% resorcinol (lotion)
3% 2-chloro-4-phenylphenol (ethanolic)
5% zinc sulfate (aqueous)

ORAL DRUGS

acetylcysteine (1·5 g daily)
chlorpheniramine (20 mg daily, Chlor-Trimeton, Schering)
griseofulvin (1 g daily, Grifulvin V, McNeil)
isoniazid (300 mg daily)
penicillin V (1 g daily, Penicillin VK, Smith-Kline)
sulfadimethoxine (1 g daily, Madribon, Roche)
sulfisoxazole (4 g daily, Gantrisin Preps, Roche)
Valium (20 mg daily, Roche)
zinc sulfate (220mg daily, Zincate, Canfield)

PHYSICAL MODALITIES

Massage (physical therapist, once daily)
PUVA (8-methoxypsoralen and UVA)
Sauna (facial, 5 min, twice daily)
Sauna (facial, 5 min + 40% sodium chloride in vanishing cream, twice daily)
Scrubs (polyester, polyethylene, pumice, borax, alumin)
UVA (thrice weekly, 20 J/cm² per dose)
UVB (thrice weekly, 2 MEDs)
Zonas tape, (applied overnight, Johnson & Johnson)

We think this focussing on the negative will help to balance the record of perfervid enthusiasm. It is too much to expect that this exposition will have much effect on the doctor's dilemma: not getting patients better is too much to bear.

These studies were not double-blind. We kept the eyes of the subjects and ourselves wide open during the tests.

By our value scheme, the agents which do not require self-deception to be perceived as effective in acne are extremely limited in number. They include: (1) oral retinoids (cis-retinoic acid); (2) oral antibiotics (tetracyclines, erythromycin); (3) topical antibiotics (clindamycin and erythromycin); (4) a single antimicrobial chemical, benzoyl peroxide; (5) comedolytic agents (salicylic acid and vitamin A acid), and with special care for special patients, corticosteroids and non-steroidal anti-inflammatory agents (ibuprofen and others). These agents should be the benchmarks for assessing the efficacy of new treatments. Greater rigorousness in conducting clinical trials will decrease false claims and save much misery and money.

University of Pennsylvania and
The Simon Greenberg Foundation,
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U.S.A.

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ALBERT M. KLIGMAN

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British Journal of Dermatology (1983) **108,** 375–378.

Correspondence

Serum levels of retinol binding protein in patients with pityriasis rubra pilaris

Sir, Finzi *et al.* have reported decreased serum levels of retinol binding protein (RBP) in all eleven of their patients with pityriasis rubra pilaris (PRP) (*British Journal of Dermatology*, 1981, **104,** 253).

This has not been our experience. In three patients with PRP whom we have studied, serum levels of RPB have been normal.

TABLE 1. Serum RBP levels in patients with PRP

Patient	Medication	Serum RBP level (mg/100 ml)
51-year-old white male	Vitamin A, (100,000 units b.d.)	5·2
55-year-old white male	none	6·0
58-year-old white male	none	5·5

Finzi *et al.* listed 7·02 ± 1·04 mg RBP/100 ml serum as being the normal range for their male patients. Goodman (1980) and Rask (1980) both report normal values of serum RBP to be about 4–5 mg RBP/100 ml serum. We have found 4–7 mg RBP/100 ml serum to be the normal range. Even using 7·02 ± 1·04 as the standard, our patients are either within normal range or just slightly below.

Based upon these findings, serum RBP levels in patients with PRP appear to be normal.

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Lichen planus and liver disease

Sir, In their interesting article on lichen planus (LP) and primary biliary cirrhosis (PBC) (*British Journal of Dermatology* 1982, **106,** 699–703), Dr Graham-Brown and his colleagues cite me as the author of an 'inconclusive report of five patients with erosive LP and cirrhotic disease'.

The aim of my cited paper (Rebora *et al.*, 1978) was to report the very high prevalence of cirrhosis (in five out of seven patients with erosive LP) irrespective of the aetiology of the liver disease. In fact, the poor general condition of some of the patients precluded the liver biopsy required for definitive diagnosis. However, enough evidence was collected to show that most of those patients were very likely to have chronic active hepatitis (CAH). I agree with Dr Graham-Brown *et al.* that some of my patients could well have had PBC, but I maintain that others had biopsy-proven CAH.

Furthermore, Dr Graham-Brown *et al.* seem to have ignored other reports associating LP with CAH (Calandra, 1972; Binet *et al.*, 1979; Monk & Pembroke, 1981). I have also reported an additional biopsy-proven CAH (1981) and in a retrospective study I found that about 10–15% of ordinary LP patients actually had biopsy-proven CAH (Rebora, Rongioletti & Canepa, 1982).

To my knowledge, my 'inconclusive report' was the first to conclude that LP, far from being a benign disease, may have a severe prognosis.

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Hepatic toxicity of ketoconazole

SIR, In his Comment article (*British Journal of Dermatology*, 1982, **106,** 597) on systemic antifungal agents, Dr D.T.Roberts suggested that vigilant supervision of liver function is necessary in patients on ketoconazole therapy. Our clinical experience with this agent in the treatment of onychomycosis fully confirms this view. During an 8-month therapeutic trial of ketoconazole (200 mg once daily) four of our thirty-six patients developed various types of liver reactions possibly induced by the drug. We wish to report briefly on these cases, emphasizing the importance of periodic liver function tests during long-term therapy.

The most severe course was seen in a 37-year-old man who after 6 months of ketoconazole therapy complained of slight epigastric pain. Physical examination was normal. Serum transaminase levels were moderately elevated but other liver function tests were within normal limits. Gastroscopy and ultrasound scan yielded normal findings. Although ketoconazole was immediately stopped serum transaminase levels rose progressively (Fig. 1). A liver biopsy showed mononuclear cell infiltration of the portal zones and abundant necrotic hepatocytes, consistent with toxic hepatitis. Prednisolone treatment (30 mg/day) was instituted whereupon biochemical abnormalities rapidly receded. Steroid treatment was continued for 5 months. Liver function tests obtained monthly thereafter have remained within normal limits.

The second case was a 40-year-old man who showed a slight increase of SGPT activity 3 months after ketoconazole therapy was started. Within another 2 months of therapy the serum enzyme level had risen to 1·4 μkat/l (normal less than 0·6 μkat/l) without any other evidence of liver dysfunction. Antibodies to mitochondria and to thyroid microsomal antigens were detected (titres 1:25 and 1:400, respectively). Serum levels of lipids, IgG and IgM as well as thyroid function tests were normal. A liver biopsy revealed a sparse inflammatory cell infiltrate of the portal zones and the hepatocyte nuclei displayed reactive features.

Upon discontinuation of therapy the transaminase concentration and titres of antibodies to thyroid antigens reversed to normal within 2 months, whereas the low titre of antibodies to mitochondria persisted.

A 51-year-old man developed a moderate and asymptomatic elevation of SGPT level (3·0 μkat/l) after 6 weeks of treatment with ketoconazole. Other liver function assays were normal and a liver biopsy was not performed. Despite continued therapy the enzyme activity became normal within 6 weeks.

The last case was a 43-year-old man who presented with abdominal pain, vomiting, jaundice and liver tenderness after 1 month in the trial. Laboratory studies showed combined cholestatic-hepatocellular damage and an eosinophilia (12%). Oral cholecystogram was normal. No liver biopsy was performed. After withdrawal of ketoconazole symptoms disappeared rapidly, although the biochemistry remained abnormal for 3 months.

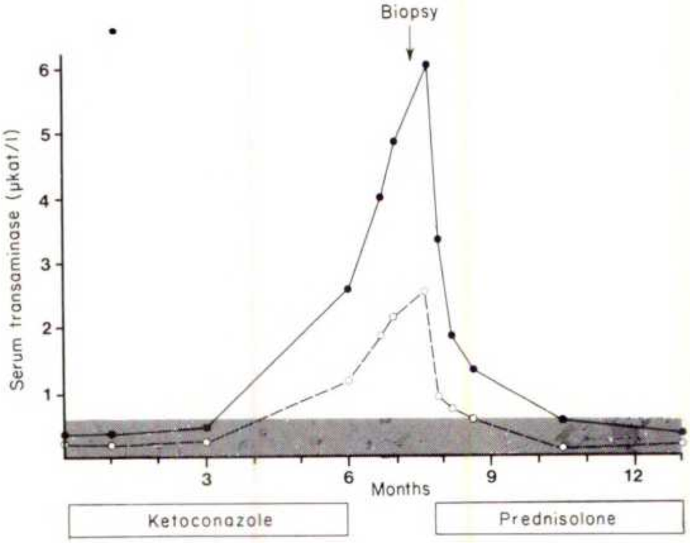

FIGURE 1. Hepatic enzyme levels after initiation of ketoconazole therapy in case 1. The shaded area represents the normal range. ●——● = alanine aminotransferase. ○– – –○ = aspartate aminotransferase.

It is difficult to be certain that the liver reactions in our patients were drug-induced. For ethical reasons we were unable to confirm a causal connection with the drug by rechallenge. Nevertheless, it should be emphasized that no patient abused alcohol, took any regular medication besides ketoconazole or had recently received a blood transfusion. In addition, acute viral hepatitis except for non-A, non-B type were reasonably excluded by serological means.

According to Janssen & Symoens (1982) transient, asymptomatic increases in serum liver enzyme levels during ketoconazole therapy are considered harmless and are no reason for stopping the drug. However, whether these biochemical alterations are accompanied or followed by any histopathological changes in the liver has not, to our knowledge, been adequately investigated. Obviously, a hepatic lesion may be present even though liver-derived enzymes show only slight aberrations, as demonstrated in our third case. Furthermore, it is not possible to predict from the first observed abnormal liver function tests whether a progressive liver reaction, as in the first case, has been initiated.

Despite its proven efficacy in severe mycoses the risk–benefit ratio in ketoconazole treatment requires careful consideration if it is to be used for long-term administration in superficial mycoses of limited severity and extent. Liver function tests before and periodically (e.g. each third or fourth week) during treatment are mandatory. In the event of abnormal values we suggest the immediate withdrawal of the drug and consideration of liver biopsy.

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REFERENCE

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British Journal of Dermatology (1983) **108,** 379.

Book Reviews

Occupational and Industrial Dermatology. H.I.Maibach and G.A.Gellin, (1982) Chicago: Year Book Medical Publishers. Pp. 375. Price £38.50.

This book consists of thirty-nine essays by thirty-five different authors. There is a lot of variation in style, readability, content and length (from four to forty-eight pages); the length not necessarily reflecting the importance of the subject. I hope it would not be entirely a matter of personal prejudice to say that it is surprising in a book of this scope that only five pages are devoted to all metals, particularly when thirty-two pages are given to biological and microbial causes and fifty-eight pages to various aspects of plastics. The book is designed for use by Health Professionals engaged in recognition, management and treatment of work-related skin diseases and is said to supplement two current books—Etain Cronin's *Contact Dermatitis* and Jean Foussereau's *Occupational Dermatology*, which means a fairly expensive dermatological package for Factory Medical Officers. The main deterrent (using it as their sole bench book) for Factory Medical Officers and Factory Inspectorate Personnel would be the incomplete and patchy coverage of the subject; for instance, no chapter on rubber and no mention of chrome ulcers; and chapters which could be really useful on work preplacement, the assessment of impairment and disability, and the role of national agencies in controlling dermatitis are all United States orientated.

If one bears it in mind as a supplement to the books mentioned, there are some outstanding chapters. For instance, it would be worth buying for the following chapters alone: allergic contact dermatitis; immunological considerations, which is one of the best expositions I have seen of the current immunological position; Langerhans cells; IA antigens; and macrophages, etc. Among the other chapters which I found useful for reference were those on pigmentation, determination of allergic contact potential chemicals, human predictive tests for allergic contact dermatitis and percutaneous absorption relative to occupational dermatology.

D.Burrows

Allergic Contact Dermatitis to Simple Chemicals—A Molecular Approach. By Gilles Dupuis and Claude Benezra (1982) New York: Marcel Dekker. Pp 183. Price SFr 105.

The main thrust of this book is an attempt to rationalize allergic contact dermatitis (ACD) from the point of view of the structures of the allergens and their ability to bind to proteins. In this context this book serves a useful purpose. It includes a large number of formulae of different classes of contact sensitizers and surveys the problems of cross sensitization in an attempt to help the toxicologist looking for inducers of ACD to anticipate the potential of certain compounds to induce ACD.

It is unfortunate that the first three chapters, which deal with the cellular aspects of ACD and the present day approaches of investigating ACD, are not only rather too brief but that the references given for further reading are in general no more recent than 1975. However, this book is a must for all dermatologists and immunotoxicologists working in the field of allergic contact dermatitis. In addition, immunologists and chemists interested in hapten-protein conjugates will find this a useful reference book to have in the library.

Darien Parker

British Journal of Dermatology (1983) **108**, 380.

News and Notices

Clinical Conference, New Hampshire, U.S.A., August, 1983

The Harvard Medical School, Department of Dermatology, will sponsor a Conference of Speciality Clinics to be held at the Mount Washington Hotel, Bretton Woods, New Hampshire, 21–27 August 1983. In these Speciality Clinics, a series of clinical problems generated by faculty and audience will be discussed by faculty and audience. Participants are invited to bring case histories, slides etc. for discussion. A daily keynote talk will be given by an expert in the area of the day's discussion. For further details, contact M.M.Bradley, Department of Dermatology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114, U.S.A.

Dermatopathology Workshop, Cleveland, October 1983

A Dermatopathology Self-Assessment Workshop will be held in Cleveland, Ohio, U.S.A. on 8 October 1983. For further information contact Wilma F.Bergfeld, M.D., 9500 Euclid Avenue, Cleveland Clinic, Cleveland, Ohio, 44106.

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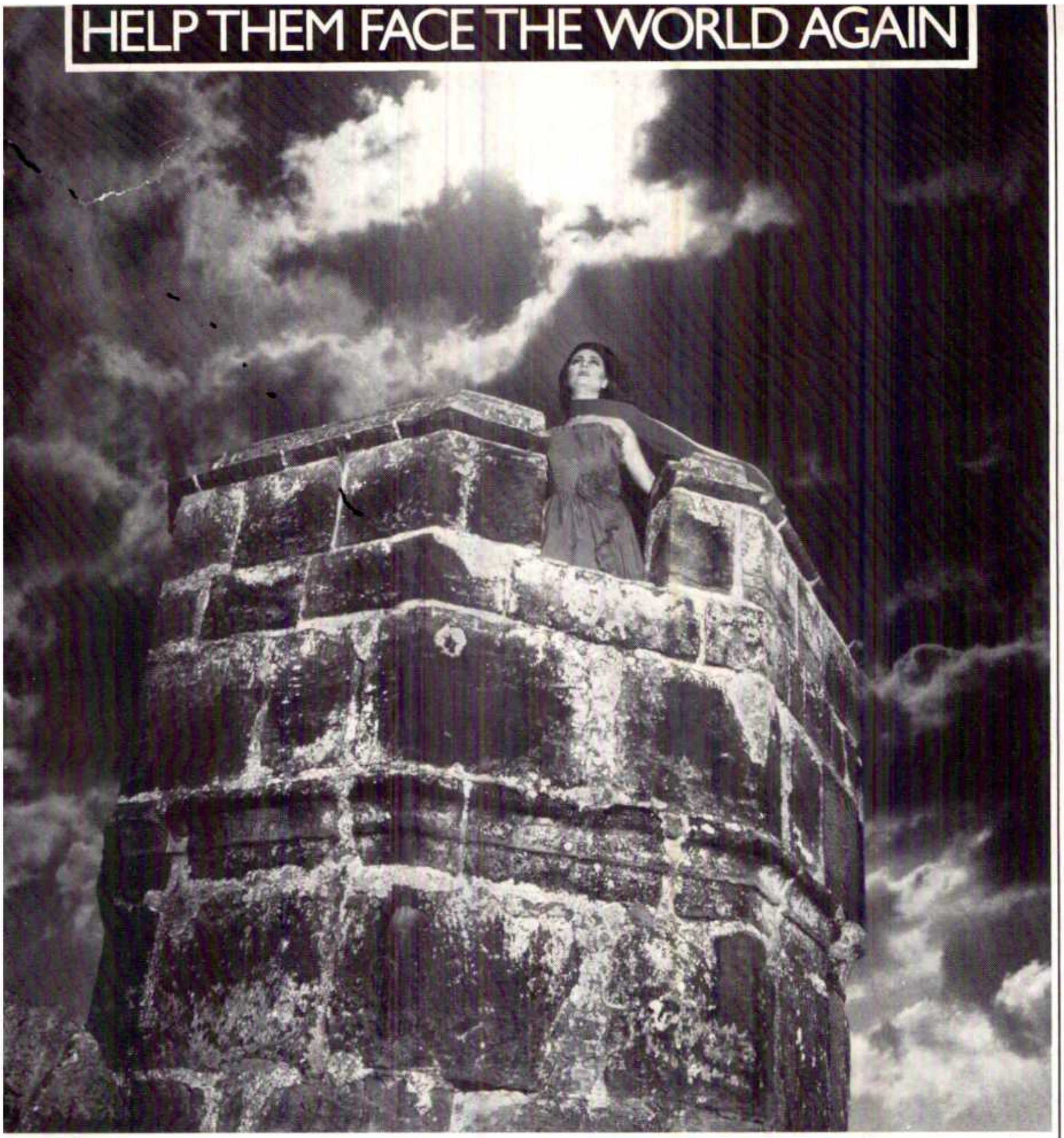

Psoriasis is a solitary condition. It has been reported that “. . . patients lives can become completely taken over by manoeuvres designed to avoid exposing their affected skin to the public eye.”[1] Alphosyl helps you bring them out of hiding with an effective and highly acceptable range of treatments.

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stimulant for the treatment of psoriasis. Alphosyl Lotion: the basic treatment for psoriasis of the body and scalp. Alphosyl Cream: designed especially for lubrication in the intertrigenous areas. Alphosyl Application PC: shampoo treatment for the scalp and dandruff. Alphosyl HC: where lo
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Rinse and repeat if necessary. CONTRA-INDICATIONS, WARNINGS ETC. For external use only. Discontinue if irritation occurs or in cases of sensitivity to coal tar. Alphosyl HC contains hydrocortisone; in infants long term continuous therapy should be avoided; adrenal suppression can occ
even without occlusion. Topical steroids should not be used extensively in pregnancy i.e. in large amounts or for long periods. PHARMACEUTICAL PRECAUTIONS: Do not refrigerate, store at room temperature. PACKAGE QUANTITIES AND BASIC NHS COST: Alphosyl Lotion 250 ml £2.
Alphosyl Cream 75 g £1.63. Alphosyl Application PC 60 g £0.50. Alphosyl HC 30 g £1.78; 45 g £2.52. PRODUCT LICENCE NOS. 0036/5008 (Lotion). 0036/5006 (Cream). 0036/5005 (Application PC). 0036/0026 (HC).
Reference: 1. Medicine 1977; 32: 18
Stafford-Miller Ltd, Professional Relations Division, Hatfield, Hertfordshire AL10 0NZ.

Lecture Notes on Dermatology

New Edition

Bethel Solomons MA, MD, FRCPI
Consultant Dermatologist to Chelmsford and Essex General Hospital, Herts and Essex General Hospital and Hertford County Hospital

From the reviews of previous editions

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'"Lecture Notes" is a useful pocket sized dermatology text book which lists and describes the most common conditions including leprosy and syphilis and has a large collection of both black and white and colour photographs which are of good quality.'
British Journal of Dermatology

The fifth edition has again been extensively revised and updated and will continue to be invaluable to students and general practitioners.

Fifth Edition, late 1982. 272 pages, 108 illustrations. Paper, about £8.50

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Dermatological Nursing and Therapy

R.H. Seville MD, FRCP, *Consultant Dermatologist*, and
E. Martin, *Senior State Enrolled Nurse, both of the Beaumont Hospital, Lancaster*

'The general nurse . . . will find this book a helpful guide . . . designed for use on the Joint Board of Clinical Nursing Studies course in dermatology . . . well illustrated, with text uncluttered by any jargon. There is an excellent dermatological formulary at the end of the book.' *Nursing Mirror*

Contents: Dermatological nursing; Psychosomatic dermatology; Psoriasis, introduction to dressings; Eczema, infantile eczema; Prurigo, neurotic, excoriations, artefacts, trichotillomania, alopecia areata, lichen planus; Seborrhoeic dermatitis, acne, rosacea, ano-genital pruritis; Urticaria and drug eruptions; Dermatitis; Immunology, blistering diseases; Cutaneous manifestations of systemic disease and malignancy; Granulomata including syphilis; Infections — bacterial, fungal and viral; Infestations; Leg ulcers; Some problems in the elderly; Tumours — benign and malignant; Procedures — biopsy, therapeutic and cosmetic; Investigations — histological, mycological and bacteriological; PUVA, X- and Grenz-ray therapy, physiotherapy, chiropody, dietetics; The dermatology team; Structure, function and care of the skin, nails and hair; Principles of physical treatment and preparations to avoid.

1981. 236 pages, 113 illustrations. Paper, £7.50

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NOTICE TO CONTRIBUTORS

The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advance in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

Papers accepted become the copyright of the Journal. This Journal is covered by *Current Contents, ISI/BIOMED, Science Citation Index* and *ASCA*.

Manuscripts should be sent to the Editor, Dr J.L.Burton, Dermatology Department, Bristol Royal Infirmary, Bristol BS2 8HW. They should be typewritten on one side of the paper only, with a wide margin, be double spaced, and bear the title of the paper, name and address of each author, together with the name of the hospital laboratory or institution where the work has been carried out. The name and full postal address of the author who will be responsible for reading the proofs should be given on the first page. A duplicate copy, complete with figures, tables and references, should be sent, since in many cases two referees are consulted. The author should keep a carbon copy of his manuscript. The Editorial Board reserves the right to make literary corrections.

Abbreviations and units. Full names with uncommon abbreviations must be given with the first mention: new abbreviations should be coined only for unwieldy names and should not be used at all unless the names occur frequently. In the title unusual abbreviations should be identified, in the introduction and discussion they should be used sparingly. Abbreviations of units should conform with those shown below.

gram(s)	g	minute(s)	min	molar	mol/l
kilogram(s)	kg	centimetre(s)	cm	millilitre(s)	ml
milligram(s) (10^{-3}g)	mg	second(s)	s	gravitational acceleration	*g*
microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm^3	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
hours(s)	h	milliequivalent	mmol		

These and other abbreviations and symbols should follow the recommendations of: *Units, Symbols and Abbreviations*, A Guide for Biological and Medical Editors and Authors (1977) The Royal Society of Medicine, London.

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There should be as few tables as possible and these should include only essential data; they should be typewritten on separate sheets and should be given Arabic numbers.

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British Journal of Dermatology, Volume 108, Number 3, March 1983

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British Journal of Dermatology

The British Journal of Dermatology is owned by and is the official organ of the British Association of Dermatologists. It is also the official organ of the Nederlandse Vereniging van Dermatologie en Venereologie (Netherlands Association for Dermatology and Venereology).

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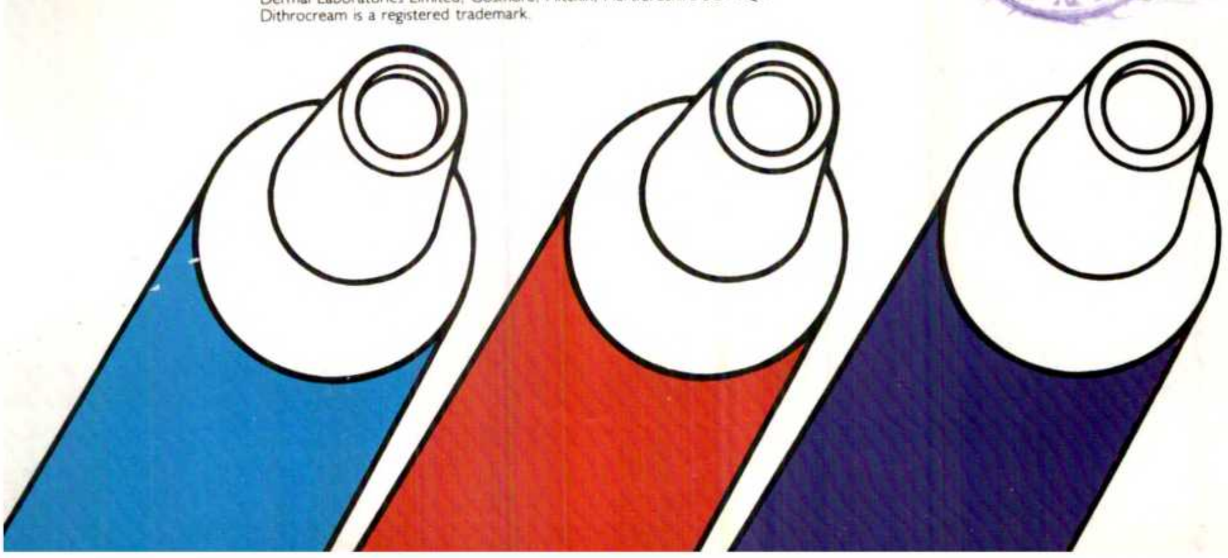

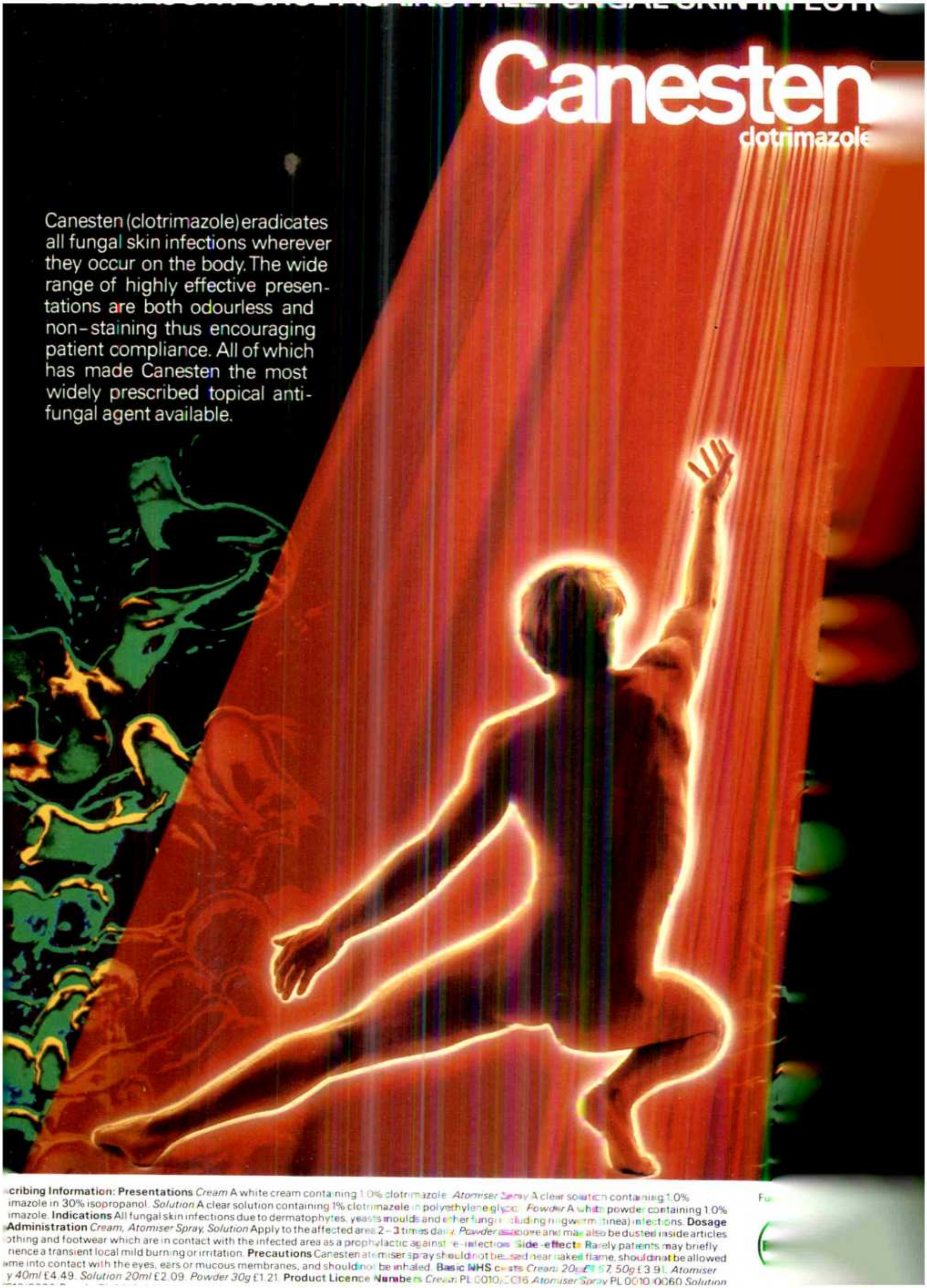

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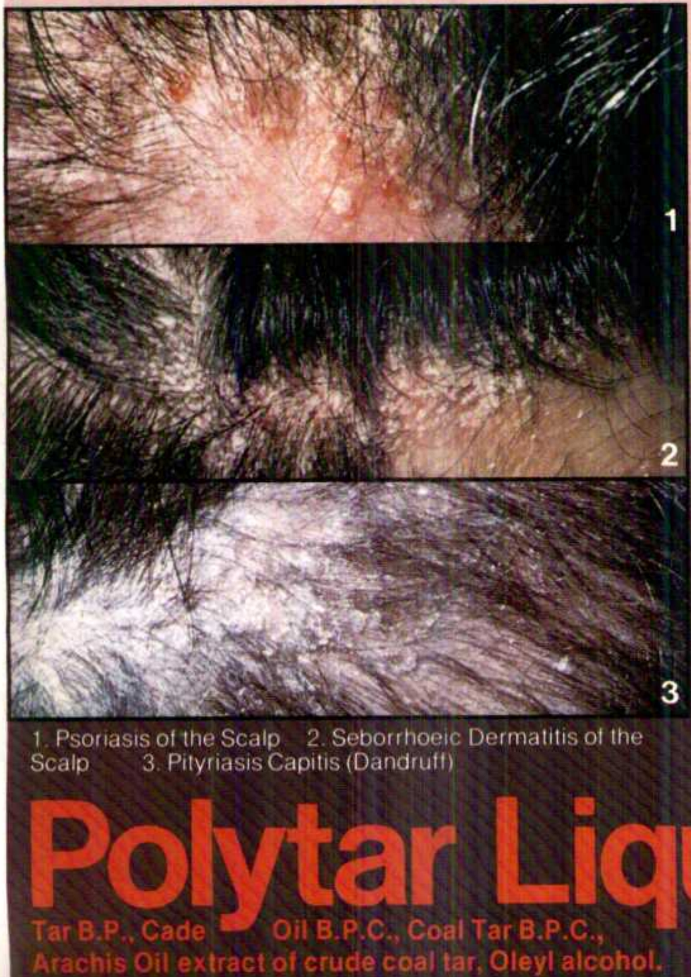

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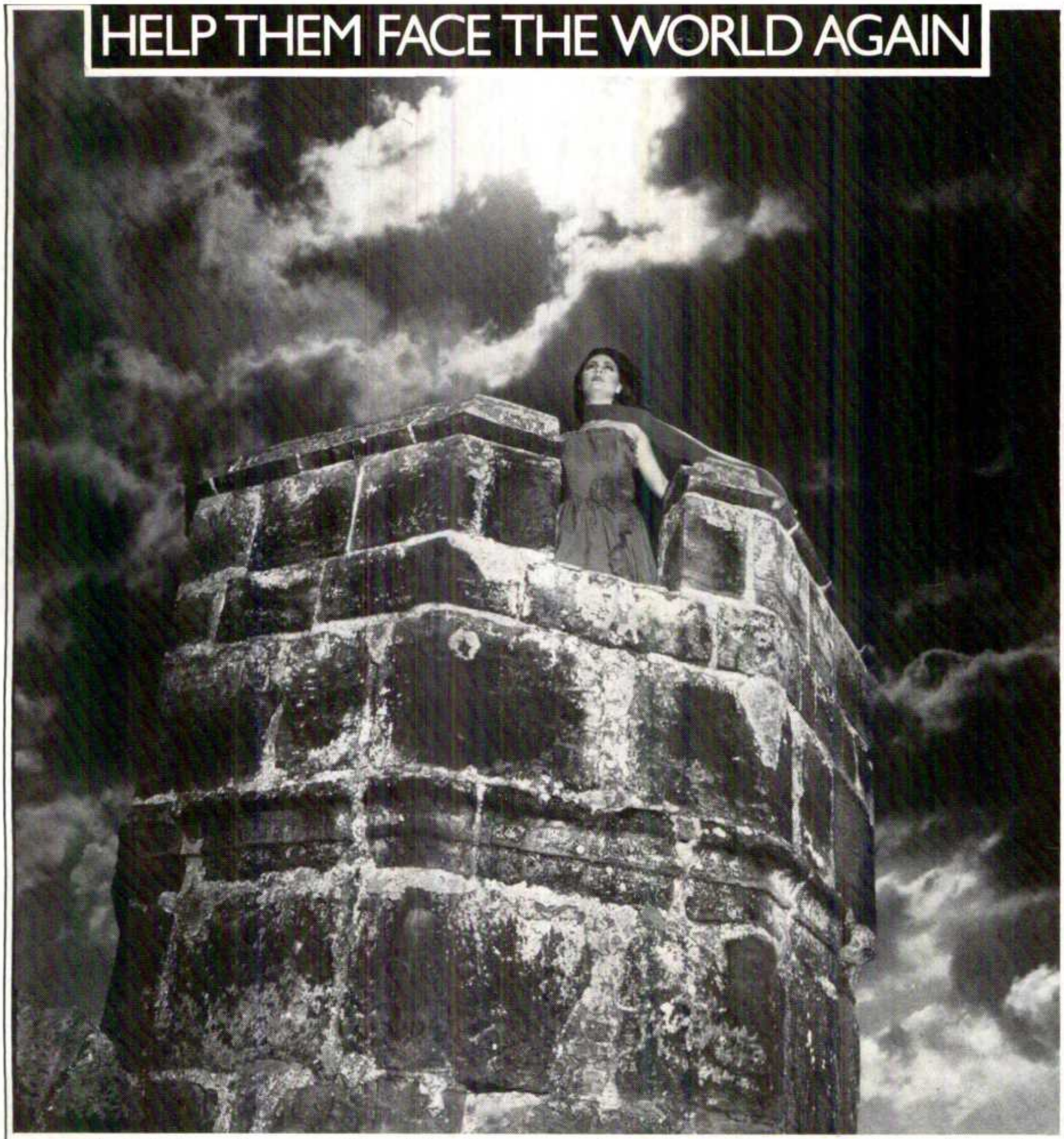

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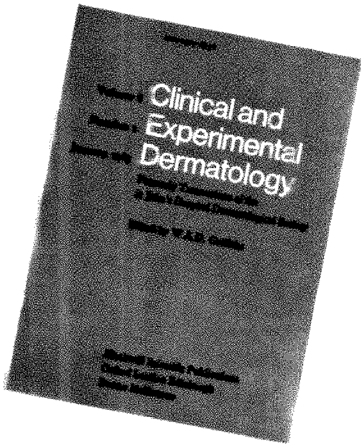

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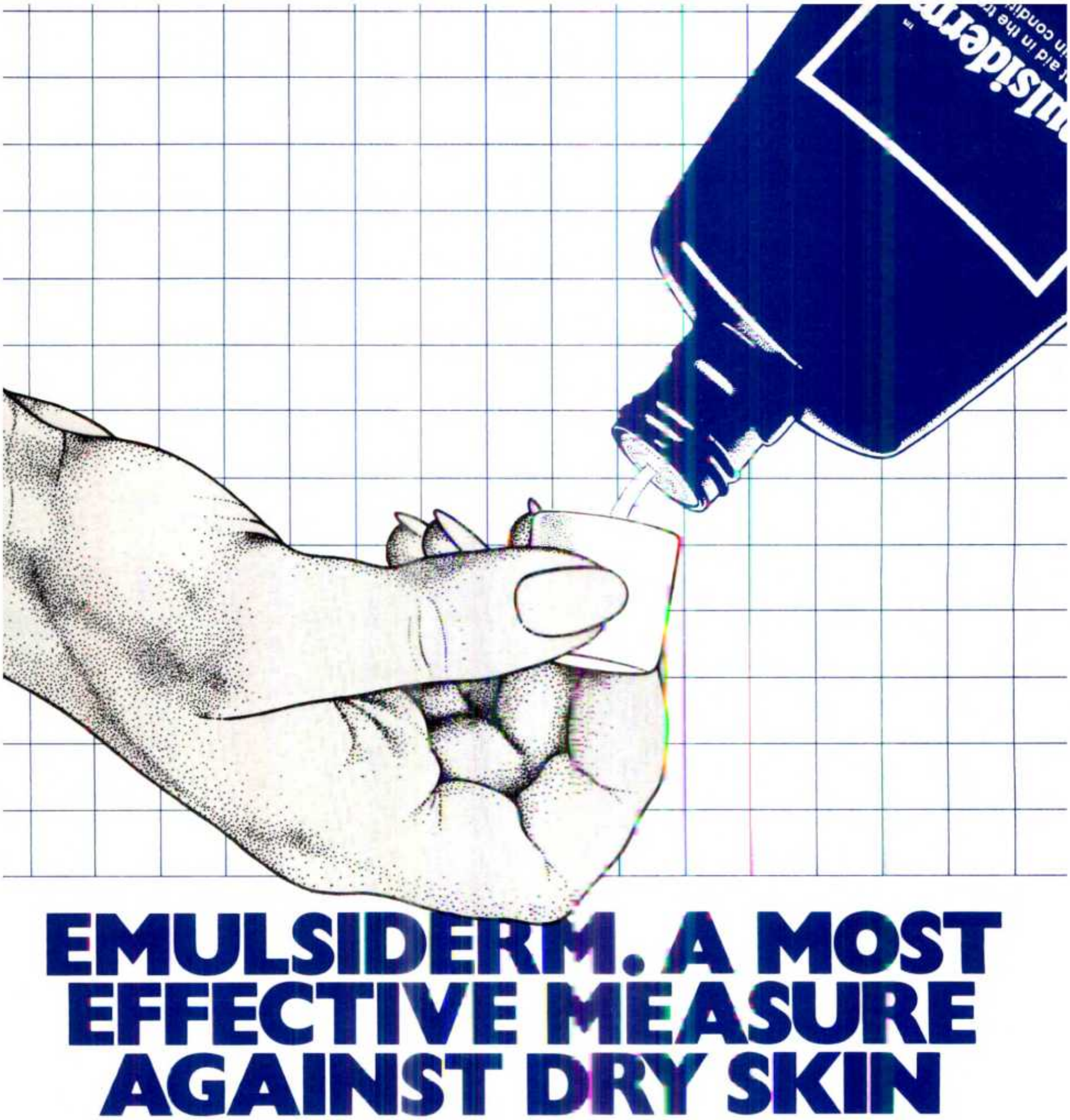

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Clinical and Laboratory Investigations

Immunological, cytochemical and ultrastructural studies in lymphomatoid papulosis

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SUMMARY

In lymphomatoid papulosis two histological types can be distinguished, i.e. type A and type B. In the present study various immunological, enzyme-histochemical and ultrastructural techniques were used to investigate the cellular infiltrate in both types of lymphomatoid papulosis. The type A lesions showed a predominance of large atypical cells, relatively few T cells and few or no Langerhans or related cells, as defined by a positive staining for OKT6 and NA 1/34 antisera. The immunological, cytochemical and ultrastructural characteristics of these large atypical cells resembled those of the Langerhans cell/interdigitating reticulum cell series. The morphology and marker profile of these large cells resembled those of Reed–Sternberg cells, which suggests a relationship between lymphomatoid papulosis type A and Hodgkin's disease. The type B lesions showed a predominance of small, medium-sized and large cerebriform mononuclear cells with the phenotype of activated T helper cells, and numerous Langerhans and/or related cells. Their cellular composition was similar to that observed in the early stages of mycosis fungoides.

Macaulay (1968) described a clinically benign but histologically malignant self-healing papular eruption which he called lymphomatoid papulosis. Because of the considerable variation in the histological descriptions of subsequently reported cases and the clinical resemblance to pityriasis lichenoides, it has been questioned whether lymphomatoid papulosis represents a distinct entity (Black, 1982). In a recent study of sixty-four skin biopsies from sixteen patients with lymphomatoid papulosis it was found that two characteristic histological types can be recognized (Willemze *et al.*, 1982a). It was shown that these two types (type A and type B), which differ not only histologically but also in clinical behaviour, are not different entities but rather represent the ends of a spectrum. The main characteristics of these two types are

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summarized in Table 1. The large atypical cells in lymphomatoid papulosis have been the subject of much controversy. Both lymphoid (Weinman & Ackerman, 1981; Burg *et al.*, 1982) and histiocytic (Black & Wilson Jones, 1972; Sandbank & Feuerman, 1972; Fuhrmans, Lange & Seifert, 1980) origin have been suggested.

In the present study various immunological, enzyme-histochemical and ultrastructural techniques were used to investigate the cellular infiltrate in both types of lymphomatoid papulosis.

METHODS

Patients

In this study skin biopsies from ten patients with lymphomatoid papulosis were investigated. All patients showed a history of recurrent, self-healing papular, nodular or plaque-like lesions. The past history and results of physical and laboratory investigations were unremarkable in all patients but one (No. 9). This patient had a history of rather persistent reddish-brown scaling plaques in the inguinal and gluteal regions for almost 25 years, clinically suggestive of parapsoriasis en plaques or the early stage of mycosis fungoides. The histological features were those of lymphomatoid papulosis type B. There was also a history of recurrent, self-healing papulonecrotic lesions, histologically lymphomatoid papulosis type A. This patient developed Hodgkin's disease of the nodular sclerosing type involving the para-aortic and para-iliac lymph nodes during the last year. A skin biopsy from a plaque-like lesions was taken prior to institution of MOPP (nitrogen mustard, vincristine, procarbazine and prednisone) chemotherapy.

Morphological examination

From every patient multiple skin biopsies were taken from papular, nodular or plaque-like skin

TABLE 1. Characteristic features of lymphomatoid papulosis, type A and type B

	Type A	Type B
Histology	Mixed cellular infiltrate with varying numbers of large atypical cells. The pattern corresponds to the age of the lesion, e.g. sparse perivascular infiltrate in early lesions, dense wedge-shaped infiltrate in fully developed lesions	Large numbers of small to large hyperchromatic CMC in perivascular infiltrates and in the basal and parabasal layers of the epidermis. No relationship between histological pattern and age of the lesion
Morphology of atypical cells	Very large cells with polymorphic, often bizarre-shaped nuclei, prominent nucleoli and abundant cytoplasm. Multinucleated variants and cells closely resembling Sternberg–Reed cells are not uncommon	Large hyperchromatic, cerebriform lymphoid cells with scant cytoplasm and inconspicuous nucleoli
Morphology of lesions	Papules, nodules	Papules, nodules and plaque-like lesions
Spontaneous involution	3–8 weeks	Weeks or months
DNA-cytophotometry	Marked aneuploid and polyploid DNA distribution pattern	Predominantly diploid DNA distribution pattern

CMC = cerebriform mononuclear cells.

lesions. For morphological examination formalin-fixed, paraplast-embedded material, as well as methyl-methacrylate embedded sections (Te Velde *et al.*, 1977) were used.

Immunological examinations

Paraffin-embedded skin biopsy specimens which had been fixed in a sublimate formaldehyde mixture (Bosman *et al.*, 1977) were stained by rabbit antisera against kappa and lambda light chains, alpha, mu and gamma heavy chains, lysozyme (all obtained from Dakopatts, Denmark), α_1-antitrypsin and α-antichymotrypsin (Behringwerke, Amsterdam) using an indirect immunoperoxidase technique. As a second step a goat-anti-rabbit IgG tagged with horseradish peroxidase, prepared in our laboratory, was used. The specificity of these antibodies and the controls of the staining procedure have previously been described (Willemze *et al.*, 1982b).

Immunofluorescence was performed on 4-μm thick cryostat sections, using an indirect immunofluorescence technique with the same antisera as mentioned above.

The presence of C3 and Fcγ-receptors was investigated by means of rosette assays on cryostat sections using ox erythrocytes coated with rabbit IgM antibody and mouse complement, or with a rabbit IgG antibody, respectively (Meijer & Lindeman, 1975; Meijer *et al.*, 1977). IgM-coated or uncoated ox erythrocytes were used as controls.

Monoclonal antibodies

A series of monoclonal antibodies directed against human thymocytes, peripheral T cells, Ia antigens and monocytes/macrophages was used. The specificities of these antisera are given in Table 2.

For demonstration of antigens reactive with these antibodies an indirect immunoperoxidase

TABLE 2. Specificity of monoclonal antibodies

Antibody	Specificity
Leu-1	95 human thymocytes, >95% peripheral blood cells some SIg$^+$ CLL cells, but not on normal B-lymphocytes (Wang *et al.*, 1980)
Leu-2a	Cytotoxic/suppressor T subset (Ledbetter *et al.*, 1981; Evans *et al.*, 1981
Leu-3a	Helper/inducer T subset (Lebetter *et al.*, 1981; Evans *et al.*, 1981)
OKT4	Helper/inducer T subset (Reinherz *et al.*, 1979)
OKT8	Cytotoxic/suppressor T subset (Reinherz *et al.*, 1980a)
OKT6	70% thymocytes (Reinherz *et al.*, 1980b); epidermal Langerhans cells (Murphy *et al.*, 1981)
Na 1/34	85% thymocytes (McMichael *et al.*, 1979); epidermal Langerhans cells (Thomas *et al.*, 1982)
HLA-DR	B lymphocytes, monocytes/macrophages, activated T cells (Warnke & Levy, 1980; Warnke *et al.*, 1980)
TA-1	100% peripheral blood T cells, 70% thymocytes, >95% peripheral blood monocytes (LeBien & Kersey, 1980)
OKM1	Peripheral blood monocytes, granulocytes, acute myelomonocytic leukemia cells (Breard *et al.*, 1980)
Anti-human monocyte 1	Peripheral blood monocytes, <25% of granulocytes, 25% of platelets (Ugolini *et al.*, 1980)

technique was used with rabbit anti-mouse IgG tagged with horseradish peroxidase (Dakopatts, Denmark) as second step. All antisera were first tested on normal tissue, i.e. lymph node, spleen, tonsil, thymus, and for NA 1/34 and OKT6 also on skin tissue. Controls were performed by replacing the monoclonal antibody with phosphate-buffered saline.

Enzyme histochemistry

Enzyme histochemistry was performed on 8 μm thick cryostat sections and included staining for acid phosphatase and α-naphthyl acetate esterase (Barka & Anderson, 1965) with incubation times of 2 h and 30 min respectively, alkaline phosphatase (Gomori, 1946) and 5-nucleotidase and adenosine triphosphatase (Wachstein & Meisel, 1957).

Electron microscopy

From skin biopsies of six patients (Nos 3, 4, 5, 6, 7 and 10) small pieces of tissue were fixed in a fixative according to McDowell & Trump (1976), postfixed in 1·5% OsO_4 in cacodylate, dehydrated in graded series of alcohol solutions and embedded in Epon 812. After selection of well-preserved pieces of tissue on semi-thin toluidin blue-stained sections, ultrathin sections were cut and stained with uranyl acetate and lead citrate.

RESULTS

From four patients (Nos 1, 2, 3 and 4) all skin biopsies showed the characteristic features of lymphomatoid papulosis type A, i.e. a mixed cellular infiltrate of lymphocytes, histiocytes and granulocytes and varying numbers of large atypical cells with vesicular nuclei, prominent nucleoli and abundant cytoplasm (Figs 1 and 2). In five patients (Nos 6, 7, 8, 9 and 10) the histological pattern and cellular composition were consistent with lymphomatoid papulosis type B, showing a predominance of small and large hyperchromatic cerebriform mononuclear cells

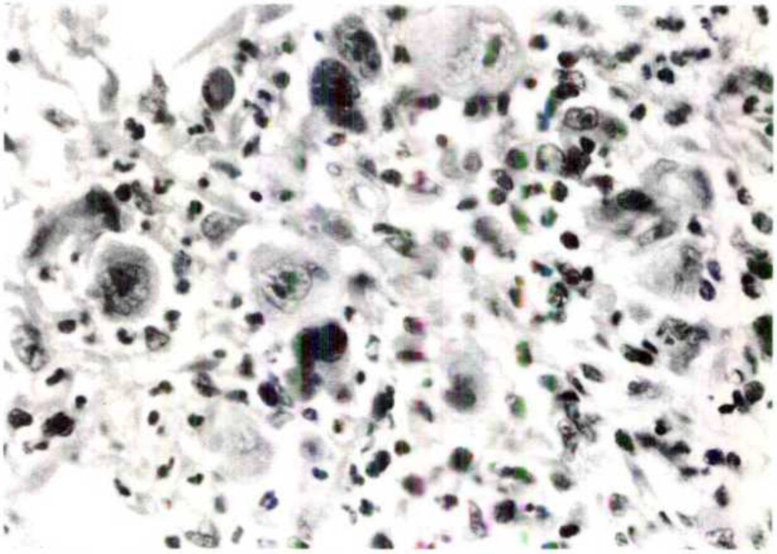

FIGURE 1. Lymphomatoid papulosis, type A. Detail of dermal infiltrate showing a mixed cellular infiltrate with very large atypical non-lymphoid cells with irregular nuclei, prominent nucleoli and abundant cytoplasm (H & E, × 400).

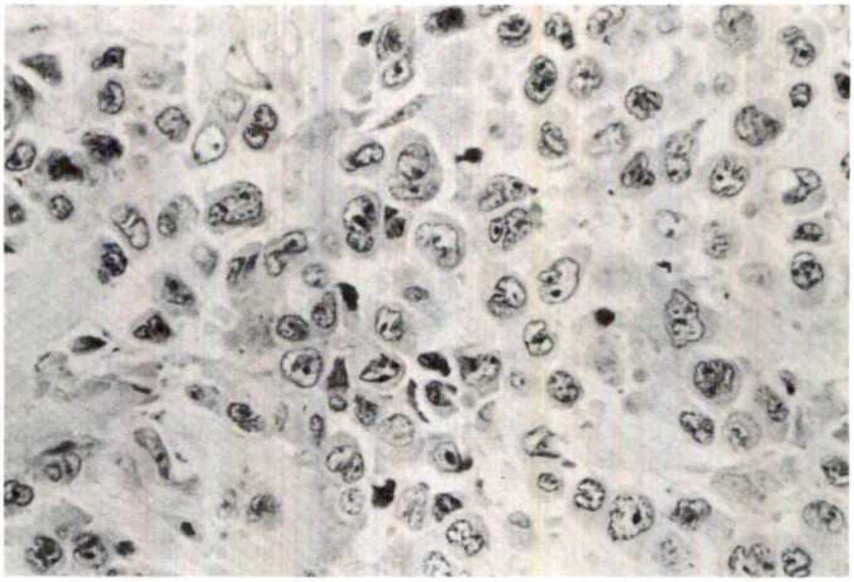

FIGURE 2. Lymphomatoid papulosis, type A. Predominance of large atypical non-lymphoid cells, which contain one or several rather small nucleoli (Giemsa, methyl-methacrylate embedded section, × 400).

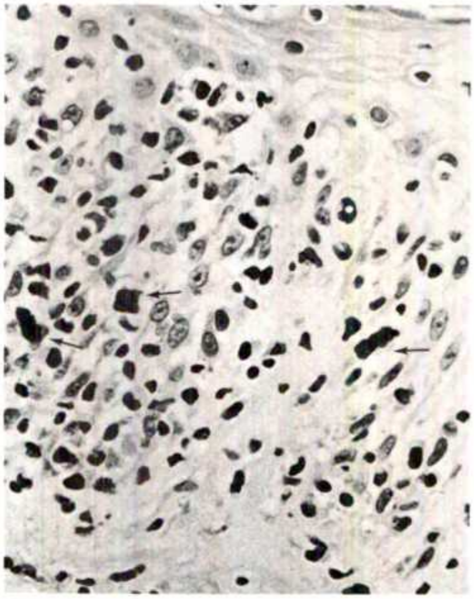

FIGURE 3. Lymphomatoid papulosis, type B. Note large atypical hyperchromatic cells in basal and parabasal layers of the epidermis (arrows), (H & E, × 320).

(CMC) which had a strong tendency to invade the basal and parabasal layers of the epidermis (Fig. 3). Patient No. 5 showed both types in different but concurrent papular lesions.

The clinical appearance, histological subclassification and the staining characteristics of the individual lesions are listed in Table 3.

Monoclonal antibodies

In all skin biopsies showing lymphomatoid papulosis type B the majority of cells stained with Leu-1 and TA-1 antibodies, indicating a predominance of T cells (Table 3). The majority of these T cells was also reactive with Leu-3a and OKT4 antisera (helper/inducer T-cell phenotype), whereas the numbers of cells reactive with Leu-2a and OKT8 antisera (suppressor/cytotoxic T-cell phenotype) ranged between 20 and 40% in all but one case (Fig. 4). In this patient (No. 7) a large nodular lesion consisted almost exclusively of cells expressing Leu-3a and OKT4 antigens, whereas only occasional Leu-2a$^+$, OKT8$^+$ cells were found scattered throughout the lesion. The large hyperchromatic CMC in these type B lesions also stained with TA-1, Leu-1, Leu-3a and OKT4 antisera. Staining with HLA-DR antiserum showed many small and large hyperchromatic HLA-DR$^-$ CMC, indicating that these represent activated T helper cells.

In the lymphomatoid papulosis type A lesions only small lymphoid cells reacted with Leu-1 and TA-1 antibodies and never exceeded 25% of the cells. The majority of these small Leu-1$^+$, TA-1$^+$ cells also expressed Leu-3a and OKT4 antigens. In contrast to most type B lesions the percentages of cells reactive with Leu-2a and OKT8 antibodies were very low (5–10%). Remarkably, in two cases (Nos 3 and 5) a proportion of the large atypical cells also stained with Leu-3a and OKT4 antisera. In the type A lesions the majority of cells in the dermal infiltrates, including the large atypical cells, were reactive with anti-HLA-DR antiserum (Fig. 5).

Staining with OKT6 and NA 1/34 antibodies showed dendritic cells in the epidermis and

TABLE 3. Results of staining with monoclonal antibodies in ten patients with lymphomatoid papulosis

Patient	Morphology of lesion	Histological type	Leu-1	Leu-2a* OKT8	Leu-3a* OKT4	TA-1	HLA-DR	OKT6* NA 1/34
1	Papule	A	+	±	+	+	+ + +	—
2	Nodule	A	+ +	+	+ +	+ +	+ + + +	occ
3	Papule	A	+ +	+	+ + +	+ +	+ + + +	occ
4	Papule	A	+ +	±	+	+ +	+ + +	occ
5	Papule	A	+ +	+	+ + +	+ +	+ + + +	ND
	Papulonecrotic	A	+ +	+	+ +	+ +	+ + + +	occ
	Papule	B	+ + +	+ +	+ + +	+ − −	+ + +	+
6	Papulonecrotic	B	+ + +	+ +	+ + +	+ − −	+ + + +	±
7	Nodule	B	+ + + +	±	+ + + +	+ − − +	+ + +	±
8	Papule	B	+ + +	+ +	+ + +	+ − −	ND	ND
9	Plaque	B	+ + + +	+ + +	+ + +	+ − − +	+ + +	±
10	Papulonecrotic	B	+ + + +	+ +	+ + +	+ − −	+ + +	+ +

Percentages semiquantitatively estimated from the total number of infiltrating cells: occ = occasional cell; ± = 1–5%; + = 6–15%, + + = 16–30%; + + + = 31–60%, + + + + = more than 60%.

* Identical staining patterns were observed with OKT8 and Leu-2a, with OKT4 and Leu-3a, and with OKT6 and NA 1/34 antisera, respectively.

ND = not done.

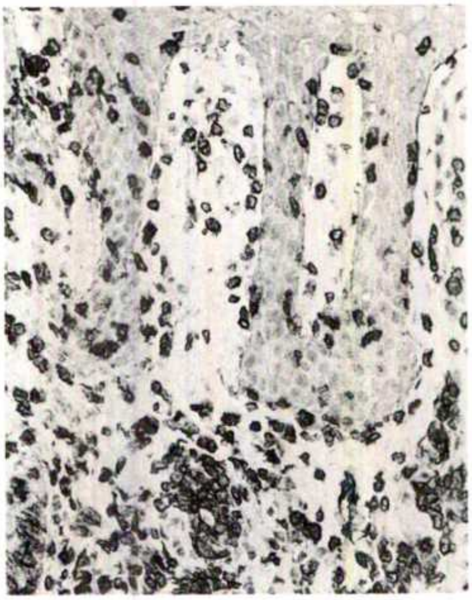

FIGURE 4. Lymphomatoid papulosis, type B. The large majority of the cells in the infiltrate and in the epidermis shows a positive staining with Leu-3a antiserum (× 176).

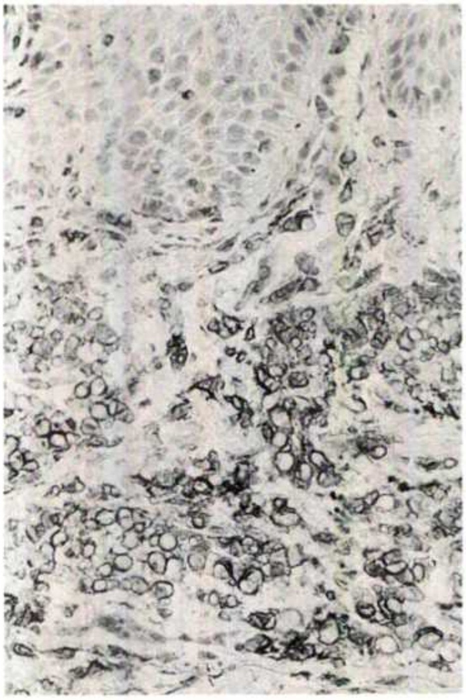

FIGURE 5. Lymphomatoid papulosis, type A, with a predominance of large atypical cells showing a positive staining with HLA-DR antiserum (× 176).

adnexal epithelia, which probably represent Langerhans cells and/or related cells. In the dermal infiltrates of the type A lesions identical cells were occasionally observed and preferentially localized in the subepidermal border. In the type B lesions however, numerous dendritic cells expressing these antigens were found scattered throughout the infiltrates (up to 15–20% of the cells) (Fig. 6). The lymphoid cells in both type A and type B lesions as well as the large atypical cells in the type A lesions were invariably non-reactive with these antibodies.

With respect to the monoclonal anti-monocyte antisera in both type A and type B lesions, OKM1 stained only occasional small mononuclear cells, whereas anti-human monocyte 1 was found to be reactive with a considerable number of both dendritic and non-dendritic cells. Occasional large atypical cells in the type A lesions showed an equivocal staining pattern when stained with this latter antiserum.

Immunofluorescence and immunoperoxidase studies

In both type A and type B lesions, staining for immunoglobulins revealed only occasional positive cells, indicating that B cells are not a major constituent of the dermal infiltrates of lymphomatoid papulosis. Examination of serial sections showed that the large atypical cells in the type A lesions were either completely negative or positive for both kappa and lambda light chains, IgA and IgG, but never for IgM, which points to an exogenous origin of these immunoglobulins. Both type A and type B lesions showed varying numbers of cells, never exceeding 20%, which stained positively for lysozyme, α_1-antitrypsin and α_1-antichymotrypsin. The large atypical cells in the type A lesions were either negative or weakly positive when stained with α_1-antitrypsin and α_1-antichymotrypsin, but invariably negative for lysozyme.

Enzyme histochemistry

In the type A lesions the large atypical cells showed a weak acid phosphatse and α-naphthyl

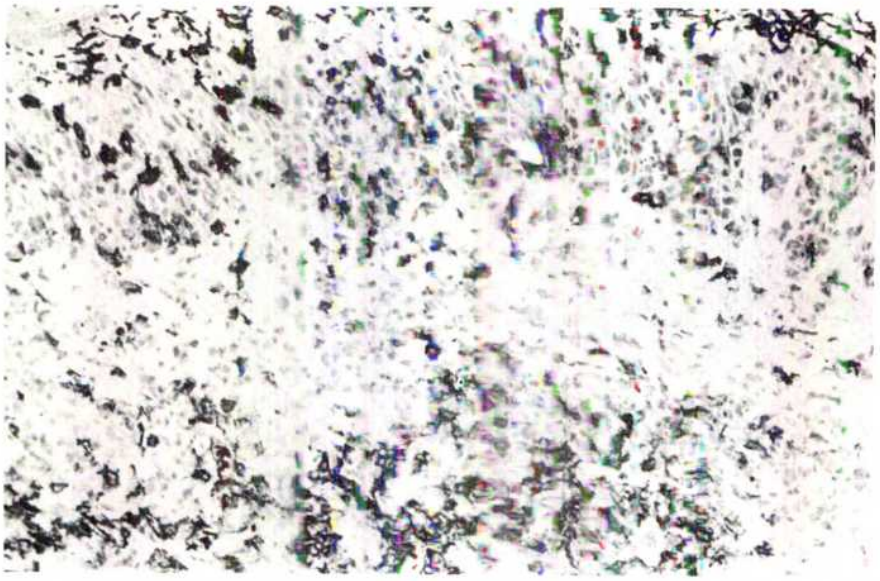

FIGURE 6. Lymphomatoid papulosis, type B. Staining with OKT6 shows numerous dendritic cells both in the epidermis and in the perivascular infiltrates, which probably represent Langerhans cells and/or related cells (× 120).

acetate esterase (ANAE) activity, but remained completely negative when stained with 5-nucleotidase and alkaline phosphatase. Varying numbers of these cells also stained for ATP-ase. In both type A and type B lesions macrophages showed a strong and diffuse staining for acid phosphatase and ANAE. In the type B lesions numerous small and large lymphoid cells showed a strong focal paranuclear activity when stained with acid phosphatase.

Rosette assays

In most cases interpretation of the results of the rosette assays was hampered by the presence of a mixed cellular infiltrate. However, in two type A lesions (Nos 4 and 5), which were almost exclusively composed of large atypical cells, a strong adherence of antibody-complement coated sheep erythrocytes (EAC-complexes) and a moderately strong adherence of rabbit-IgG coated sheep erythrocytes (EA-complexes) were observed, indicating the presence of both C3 and Fcγ receptors on these large atypical cells.

Electron microscopy

In the type A lesions a predominance of medium-sized to large non-lymphoid cells were observed, characterized by very irregularly-shaped nuclei, abundant clear cytoplasm and a ruffled cell surface and varying numbers of lymphocytes, including CMC, eosinophils and granulocytes. The nuclei of the non-lymphoid cells which were much larger than those of surrounding lymphocytes, showed deep indentations and lobulations, a very thin rim of heterochromatin and one or several prominent nucleoli (Fig. 7). The cytoplasm contained a well-developed Golgi apparatus, several small lysosomes and mitochondria and occasional phagolysosomes filled with electrondense granular material. Rough endoplasmic reticulum and ribosomes were sparse. Birbeck granules were not found. The bizarre nuclei of these cells were reminiscent of those of interdigitating reticulum cells (IDC). However, unlike IDC they did not

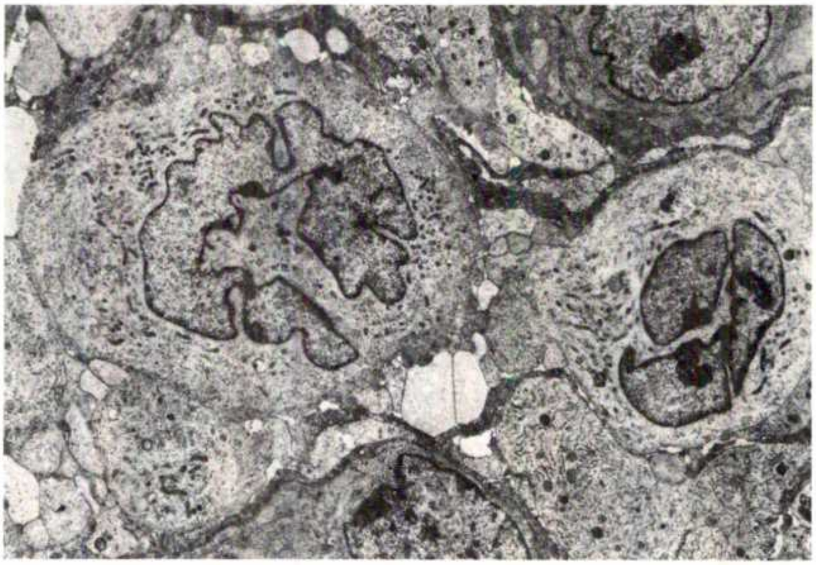

FIGURE 7. Lymphomatoid papulosis, type A. Electron micrograph showing large atypical non-lymphoid cells with very irregularly-shaped nuclei, resembling those of interdigitating reticulum cells (Uranyl acetate and lead citrate, × 2100).

have a characteristic tubulovesicular system, and only occasionally showed cytoplasmic invaginations and indentations.

The type B lesions were predominantly composed of lymphoid cells, including many medium-sized and large CMC, which were preferentially located at the dermal-epidermal junction. These were characterized by a high nucleus to cytoplasm ratio, deep and narrow nuclear indentations, condensed chromatin along the nuclear membrane and a cytoplasm poor in organelles (Fig. 8). In addition, varying numbers of non-lymphoid cells similar to those in the type A lesions, but usually smaller in size, were observed.

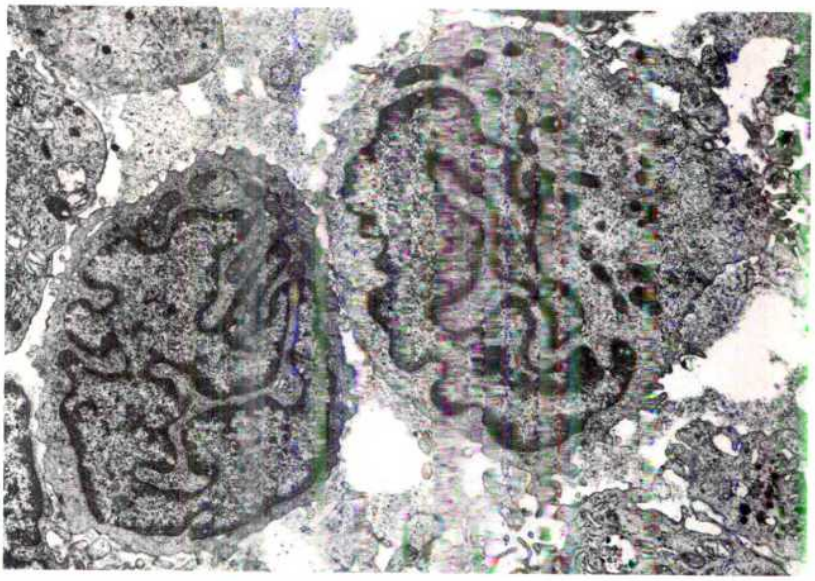

FIGURE 8. Lymphomatoid papulosis, type B. Electron micrograph showing two moderately large lymphoid cells with deep nuclear indentations (cerebriform mononuclear cells). (Uranyl acetate and lead citrate, × 5400).

DISCUSSION

The results of the present study underline our previous findings (Willemze *et al.*, 1982a) that lymphomatoid papulosis can be divided into two different histological types, i.e. type A and type B.

The cellular composition of these two types differed considerably. The dermal infiltrates in the type A lesions showed varying numbers of very large atypical cells, relatively few cells reactive with the pan-T cell antisera TA-1 and Leu-1 (25% or less) and no or only occasional Langerhans cells and/or related cells, as defined by a positive staining for OKT6 and NA 1/34 antibodies (Table 3). Only occasional cells expressed surface or intracytoplasmatic immunoglobulins, indicating that the number of B cells in these infiltrates is negligible.

In contrast to the type A lesions, the type B lesions were predominantly composed of Leu-1$^+$, TA-1$^+$ cells. The majority of these T cells, including large hyperchromatic CMC, were also stained by OKT4 and Leu-3a antisera, which define a helper/inducer T-cell subset. The predominance of a T helper population in these type B lesions was supported by the results of enzyme histochemistry showing a paranuclear acid phosphatase activity in a significant proportion of these cells. The high percentages of cells that were stained by anti-HLA-DR antiserum, together with the presence of only occasional B cells and relatively low numbers of macrophages indicate that most of these T cells also expressed Ia-like antigens and therefore represent activated T helper cells. The type B lesions differed also from the type A lesions in that they contained numerous OKT6$^+$, NA 1/34$^+$ Langerhans and/or related cells (up to 20%).

The type A lesions contained varying numbers of very large atypical cells with polymorphous, often bizarre-shaped nuclei, prominent nucleoli and abundant cytoplasm, which sometimes closely resembled Sternberg–Reed cells. Multinucleated variants were not uncommon. Immunofluorescence and immunoperoxidase studies showed a negative or polyclonal staining for surface and intracytoplasmatic immunogloublins, indicating that these cells are not of B-cell origin. Moreover, they were not reactive with any of the monoclonal anti-T cell antisera in all but two cases (Nos 3 and 5), in which a proportion of these cells was stained by Leu-3a and OKT4 antisera, which define a helper/inducer T-cell subset. This does not necessarily imply that these cells represent T helper cells, since Leu-3a antiserum also reacts with antigens present on some cells of monocyte/macrophage lineage (Dvoretsky *et al.*, 1982) and malignant Langerhans cells (Oseroff *et al.*, 1982). Similarly, OKT4 antiserum is reactive with histiocytosis X cells (Harrist *et al.*, 1982). In our view the ultrastructural findings, the presence of C3 and Fcγ-receptors, and the strong expression of Ia-like antigens are more in favour of a histiocytic origin of these cells. However, tissue macrophages and the neoplastic cells in true histiocytic lymphomas or histiocytic sarcomas (Van der Valk *et al.*, 1981; Willemze *et al.*, 1982b) and their disseminated form malignant histiocytosis (Meister, Huhn & Nathrath, 1980; Meister & Nathrath, 1980) show strong and diffuse acid phosphatase and α-naphthyl acetate esterase activity and varying amounts of lysozyme, α_1-antitrypsin and α_1-antichymotrypsin. Moreover, the large majority of these cells express monocyte-associated antigens as demonstrated by the monoclonal antibodies TA-1, OKM1 and anti-human monocyte 1 (Van der Valk *et al.*, 1982). In contrast, the large atypical cells in the lymphomatoid papulosis type A lesions showed only low contents of acid phosphatase and α-naphthyl acetate esterase with varying degrees of ATP-ase activity and a negative or only weakly positive staining with α_1-antitrypsin and α_1-antichymotrypsin. They were invariably negative when stained with lysozyme and the monoclonal anti-monocyte antibodies TA-1, OKM1 and anti-human monocyte. This staining pattern, together with the presence of C3 and Fcγ-receptors and Ia-like antigens suggests that these cells are closely related to a subpopulation of monocyte-derived cells, which includes Langerhans cells, indeterminate cells, veiled cells and interdigitating reticulum cells (Hoefsmit, Duyvestyn & Kamperdijk, 1982). The negative staining with OKT6 and NA 1/34 antisera and the absence of characteristic Birbeck granules show that these cells are not Langerhans cells.

Although the type A and the type B lesions are considered to belong to the same spectrum, the results of the present study clearly showed clearcut differences between the characteristic type A and type B lesions. The cellular composition of the type B lesions, showing a predominance of small, medium-size and large hyperchromatic CMC with the phenotype of (activated) T helper cells and numerous Langerhans cells and/or related cells, is similar to that in the early stages of mycosis fungoides (Thomas *et al.*, 1982), but it has also been found in benign inflammatory dermatoses such as atopic dermatitis and chronic eczema, histologically characterized by a band-like mononuclear infiltrate (Willemze *et al.*, 1983). The type A lesions, however, showing a predominance of large atypical cells with a marker profile resembling that of cells of the Langerhans cell/interdigitating reticulum cell series, relatively few T cells and few or no Langerhans cells, do not seem to have any resemblance to mycosis fungoides. The morphological appearance of these large atypical cells, which can closely resemble true Sternberg–Reed

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The cell population of cutaneous B-cell lymphomas

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SUMMARY

The cellular composition of the dermal infiltrates of eleven patients with a cutaneous B-cell lymphoma (four centroblastic lymphomas, two centroblastic/centrocytic lymphomas and five immunocytomas) was investigated. The distribution of both the neoplastic and the non-neoplastic cells (reactive T cells, macrophages and dendritic reticulum cells) in primary and secondary cutaneous B-cell lymphomas was very similar to that of B-cell lymphomas of the same type in lymph nodes. Reactive T cells and dendritic reticulum cells were only occasionally found in centroblastic lymphoma, but were very numerous in centroblastic/centrocytic lymphoma. The large majority of these T cells in centroblastic/centrocytic lymphoma showed the phenotype of activated T-helper cells (Leu-1$^+$, Leu-3a$^+$, OKT4$^+$, HLA-DR$^+$). In immunocytomas many T cells reactive with Leu-1, Leu-3a, and OKT4 but not with anti-HLA-DR antiserum, and varying numbers of dendritic reticulum cells were found. Since B-cell lymphomas in lymph nodes are the neoplastic counterparts of B-cell reactions which take place after antigenic stimulation in the different lymph node compartments, our results suggest that cutaneous B-cell lymphomas may be the malignant counterparts of similar B-cell reactions in the skin.

It has been suggested that non-Hodgkin lymphomas are the neoplastic counterparts of reactions which normally take place after antigenic stimulation in specific compartments of the lymph node, i.e. the follicle centre cell reaction in the follicular compartment, the plasma cell reaction in the medullary cords, and the specific T-cell reaction in the paracortical area (Lukes & Collins, 1973; Lennert, 1973; Lennert, 1978; Mann, Jaffe & Berard, 1979). In these reactions macrophages, modulatory T cells and specific antigen-presenting cells (dendritic reticulum

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cells for B cells, and interdigitating reticulum cells for T cells) are considered to play a regulatory role (Lennert, Kaiserling & Müller-Hermelink, 1978; Lennert & Stein, 1982).

Recent studies of the distribution of dendritic reticulum cells and modulatory T cells in non-Hodgkin lymphomas of B-cell origin in lymph nodes (Lennert *et al.*, 1978; Stein *et al.*, 1981; Stein *et al.*, 1982) have shown large numbers of T cells and dendritic reticulum cells in follicular centroblastic/centrocytic lymphoma; varying numbers of dendritic reticulum cells, but few or no T cells in diffuse centrocytic lymphoma; and few dendritic reticulum cells and T cells in centroblastic lymphoma. In immunocytomas, varying numbers of T cells but few dendritic reticulum cells were found (Kaiserling, 1977; Stein *et al.*, 1981).

With regard to the cutaneous B-cell lymphomas, investigations have been directed only at characterization of the neoplastic B cells (Braun-Falco, Burg & Schmoeckel, 1981; Kerl, Rauch & Hödl, 1982). No data are available regarding the distribution of dendritic reticulum cells and modulatory T cells in the skin infiltrates in these lymphomas.

In the present study we have investigated the distribution of the neoplastic and non-neoplastic cells in the dermal infiltrates of eleven patients with a cutaneous B-cell lymphoma. The results show that the cellular composition of primary and secondary cutaneous B-cell lymphomas is very similar to that of B-cell lymphomas localized in lymph nodes. These findings suggest that primary extralymphatic B-cell lymphomas are the neoplastic counterparts of B-cell reactions which, after antigenic stimulation, apparently can also take place in an extralymphatic organ such as the skin.

METHODS

Patients

Eleven patients with a cutaneous B-cell lymphoma who were referred to our department during the last 2 years were selected for this study. All cases were classified according to the criteria of the Kiel classification (Gérard-Marchant *et al.*, 1974; Lennert, Stein & Kaiserling, 1975) showing centroblastic lymphoma in four cases (Nos 1, 2, 3 and 4), centroblastic/centrocytic in two cases (Nos 5 and 6) and immunocytoma in five cases (Nos 7, 8, 9, 10 and 11). Two out of four centroblastic lymphomas (Nos 2 and 3) had developed from centroblastic/centrocytic lymphoma after a period of 5 and 2 years respectively. Patient No. 3 presented in early 1980 with multiple skin lesions, but at first admittance wide-spread centroblastic/centrocytic lymphoma with involvement of lymph nodes, liver, spleen, bone marrow and skin was demonstrated. Several chemotherapeutic regimens had successfully affected the lymph node and visceral localizations, but failed to control the cutaneous involvement. Recent skin and lymph node biopsies revealed centroblastic lymphoma. The past history of patient No. 11 showed a malignant lymphoma of the left ethmoid 6 years prior to the cutaneous involvement, which had successfully been treated with radiotherapy. Moreover, bone marrow involvement was demonstrated in this patient at the time of skin biopsy. In the remaining nine patients thorough physical and laboratory investigations including bone marrow biopsy, chest radiography, liver and spleen scintigraphy and lymphography failed to demonstrate extracutaneous involvement at the time of skin biopsy. In three patients (Nos 7, 9 and 10) a monoclonal increase of serum IgM was observed. The clinical details and follow-up data are summarized in Table 1.

Morphological examinations

From each patient multiple skin biopsy specimens were obtained from untreated skin lesions. For morphological examination, formalin-fixed paraplast embedded material, methyl-methac-

TABLE 1. Clinical data of eleven patients with a cutaneous B-Cell lymphoma

Patient	Sex	Age	Classification	P/S*	Clinical features	Initial therapy	Extracutaneous involvement	Therapy	Survival†
1	F	84	Centroblastic	P	Multiple tumours both lower legs	RT	Inguinal lymph nodes after 6 months	RT	Died 8 months after onset
2	M	57	Centroblastic‡	P	Multiple tumours on the trunk	Excision	Supraclavicular lymph node after 6 months	COP	CR, 12 months
3	M	70	Centroblastic§	S	Plaques and tumours on trunk and scalp	COP	Axillary lymph nodes	COP	8 months; persistent lymphoma
4	F	59	Centroblastic	P	Large ulcerating tumours on chest	COP	None	—	8 months
5	M	47	Centroblastic/ centrocytic	P	Large tumours on the scalp	Excision	Para-aortic lymph nodes after 2 years	ACOP	CR, 36 months
6	F	76	Centroblastic/ centrocytic	P	Solitary tumour abdomen	Excision	None	—	CR, 14 months
7	F	79	Immunocytoma	P	Solitary tumour right upper leg	Excision	None	—	Died after 6 Months from unrelated disease
8	M	56	Immunocytoma	P	Solitary tumour left upper arm	RT	None	—	CR, 14 months
9	M	23	Immunocytoma	P	Multiple tumours face and arms	RT	None	—	Unknown
10	F	65	Immunocytoma	P?	Nodules and plaques on face, trunk and limbs	—	Splenomegaly after 5 weeks		COP CR, 20 months
11	M	54	Immunocytoma¶	S	Nodules on back	Excision	Bone marrow at the time of skin biopsy	—	12 months; slowly progressive lymphoma

RT = radiotherapy; CR = complete remission; (A) COP = (Adriamycin), Cyclophosphamide, Oncovin, Prednisone

* P/S: primary (P) or secondary (S) cutaneous involvement

† Survival expressed in months after diagnosis

‡ Preceded by centroblastic/centrocytic lymphoma limited to the skin for at least 5 years

§ Preceded by centroblastic/centrocytic lymphoma with widespread cutaneous, lymph node and visceral involvement for two years

¶ Past history of immunocytoma of left ethmoid six years prior to cutaneous involvement rylate embedded sections (Te Velde *et al.*, 1977) and in some cases routinely processed semithin sections were used.

Immunological examinations

Surface immunoglobulins (SIg) were demonstrated by an indirect immunofluorescence technique on cryostat sections using rabbit antisera directed against IgA, IgD, IgG and IgM heavy chains and kappa and lambda light chains (Dakopatts, Denmark). Paraffin-embedded skin biopsy specimens fixed in a sublimate formaldehyde mixture (Bosman *et al.*, 1977) were used for demonstration of intracytoplasmic immunoglobulins (CIg), J chain, lysozyme, alpha$_1$-antitrypsin and alpha$_1$-antichymotrypsin, using the PAP technique (Taylor & Mason, 1974). The specificities of these antisera have previously been described (Willemze *et al.*, 1982). C3 and Fcγ receptors were demonstrated by means of a rosette assay on cryostat sections as described previously (Meijer & Lindeman, 1975; Meijer *et al.*, 1977).

Enzyme histochemistry

Eight-μm thick cryostat sections were used for all enzyme stainings. Adenosine triphosphatase (ATP-ase) and 5-nucleotidase were demonstrated by the method of Wachstein & Meisel (1957) with incubation times of 1½ h and 2 h respectively. Alphanaphthyl acetate esterase (ANAE) was demonstrated by Gomori's method as modified by Barka & Anderson (1965), with 30 min of incubation. Acid and alkaline phosphatase staining was performed according to Gomori (1946, 1952) using β sodium glycerophosphate as a substrate with an incubation of 2 h.

Monoclonal antibodies

A series of monoclonal antibodies reactive with T-lymphocyte surface antigens was used. These included Leu-1, Leu-2a, Leu-3a, HLA-DR (Becton Dickinson, FACS Systems, Sunnyvale, CA), OKT4, OKT6, OKT8 (Ortho Pharmaceuticals, Ltd. Raridan, NY), Na 1/34 (Sera-Lab., England and TA-1 (kindly given by Dr J. Jansen). In addition two monoclonal antibodies raised against a pre-B ALL cell line, BA-1 and BA-2 (kindly provided by Dr. J. Kersey) were used. The specificities of these antibodies are given in Table 2.

TABLE 2. Specificity of monoclonal antibodies

Antibody	Specificity
Leu-1	95% human thymocytes, >95% peripheral blood cells, some SIg$^+$ CLL cells, but not on normal B-lymphocytes (Wang *et al.*, 1980)
Leu-2a	Cytotoxic/suppressor T-subset (Ledbetter *et al.*, 1981; Evans *et al.*, 1981)
Leu-3a	Helper/inducer T-subset (Ledbetter *et al.*, 1981; Evans *et al.*, 1981)
OKT4	Helper/inducer T-subset (Reinherz *et al.*, 1979a, b)
OKT8	Cytotoxic/suppressor T-subset (Reinherz *et al.*, 1979a; 1980a)
OKT6	70% thymocytes (Reinherz *et al.*, 1980b); epidermal Langerhans cells (Murphy *et al.*, 1981)
Na 1/34	85% thymocytes (McMichael *et al.*, 1979); epidermal Langerhans cells (Thomas *et al.*, 1982)
HLA-DR	B-lymphocytes, monocytes/macrophages, activated T-cells (Warnke & Levy, 1980; Warnke *et al.*, 1980)
TA-1	100% peripheral blood T-cells, 70% thymocytes, >95% peripheral blood monocytes (LeBien & Kersey, 1980)
BA-1	Non-SIg, Non-HLA-DR structure on 80% of ALL; all SIg$^+$ CLL cells and >80% SIg$^+$ non-Hodgkin's lymphomas; 6–10% peripheral B lymphocytes (Abramson, Kersey & LeBien, 1981)
BA-2	65% ALL; some SIg$^+$ CLL, but not on peripheral B lymphocytes (Kersey *et al.*, 1981)

For *in situ* demonstration of antigens reactive with the aforementioned antibodies a four-step immunoperoxidase technique was applied on 6-μm thick cryostat sections, as previously described (Poppema *et al.*, 1981; Willemze *et al.*, 1983).

RESULTS

Histological features

In all cases a dense dermal infiltrate was separated from an intact epidermis by a distinct grenz zone. The distribution of the infiltrate was either diffuse, extending from the upper dermis into the subcutaneous fat (Nos 1, 2, 4, 5, 6 and 7), or primarily perivascular and periadnexal with the tendency to coalesce in the middle and deep dermis (Nos 3, 8, 9, 10 and 11). In three cases with centroblastic lymphoma (Nos 1, 2 and 4) the dermal infiltrate consisted predominantly of centroblasts with only a few scattered centrocytes, B-immunoblasts and reactive histiocytes (Fig. 1). Mitotic figures were numerous (mean: 3–5/HPF, × 500). In case 3 both areas with equal numbers of centroblasts and centrocytes and areas almost wholly consisting of centroblasts were found. In centroblastic/centrocytic lymphoma many small and medium-sized centrocytes, varying numbers of centroblasts (5 to 10% of the cells) and small lymphocytes were observed. The dermal infiltrates of the patients with immunocytoma showed a predominance of lymphocytes and lymphoplasmacytoid cells (Fig. 2), intermingled with varying numbers of plasma cells, blast cells (centroblasts, B-immunoblasts and plasmablasts), centrocytes and mast cells. The infiltrates of two patients (Nos 7 and 8) contained large numbers of plasma cells and therefore represent the lymphoplasmacytic subtype (Fig. 3). The presence of a considerable number of blast cells in two cases (Nos 10 and 11) was consistent with the polymorphic subtype of immunocytoma. In case 9, which represented the lymphoplasmacytoid subtype, some areas showed a rather large number of centrocytes. In all five cases cells with intranuclear and/or intracytoplasmic PAS-positive globular inclusions were observed (Fig. 4).

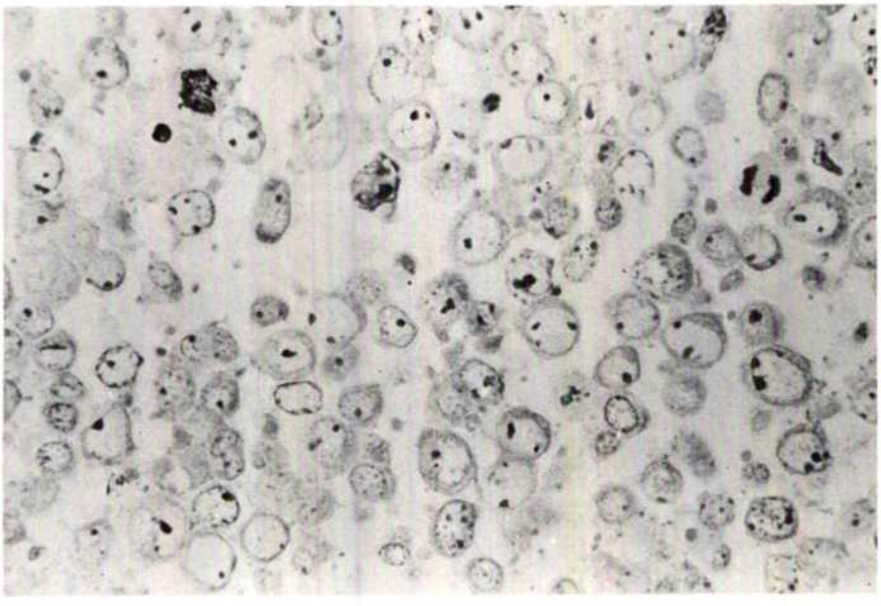

FIGURE 1. Centroblastic lymphoma (patient No. 1). Uniform proliferation of centroblasts (Giemsa (methyl-methacrylate embedded section), × 1000).

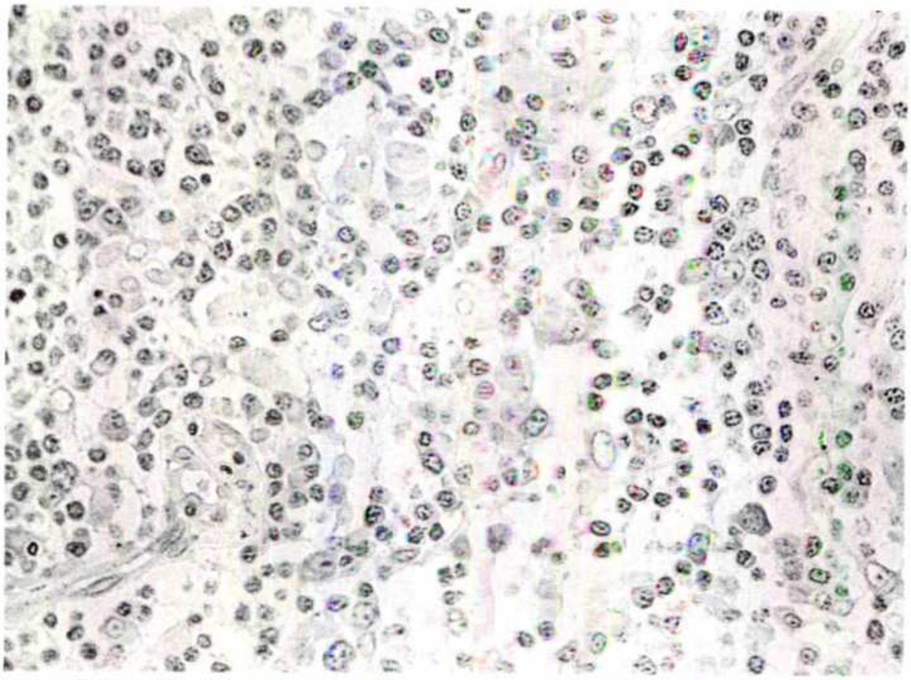

FIGURE 2. Lymphoplasmacytoid subtype of immunocytoma (patient No. 9). Many lymphoplasmacytoid cells among lymphocytes (Giemsa (methyl-methacrylate embedded section), × 550).

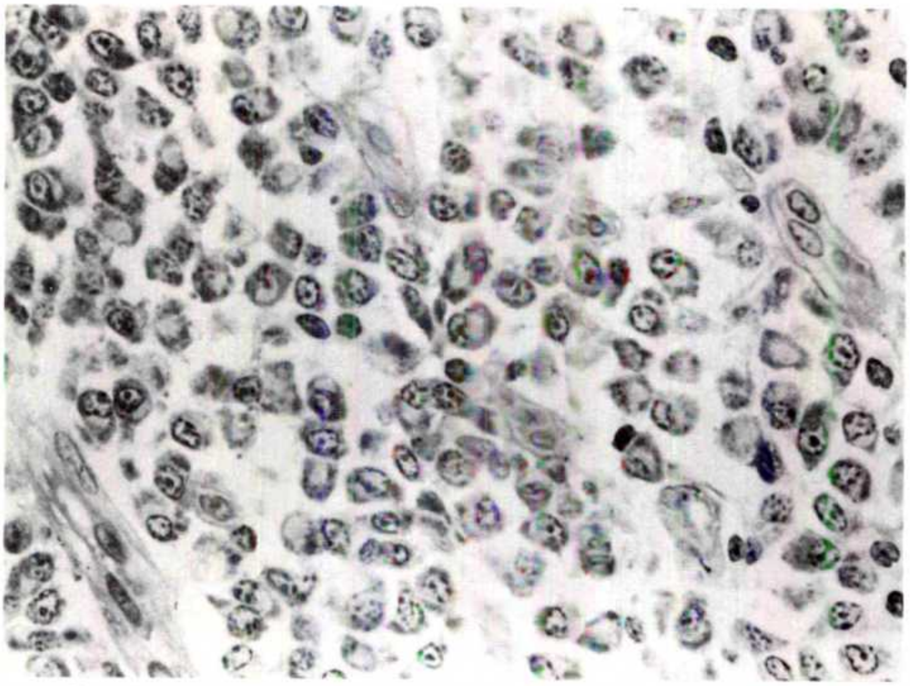

FIGURE 3. Lymphoplasmacytic subtype of immunocytoma, showing numerous plasma cells (Patient No. 7) (Giemsa (methyl-methacrylate embedded section), × 800).

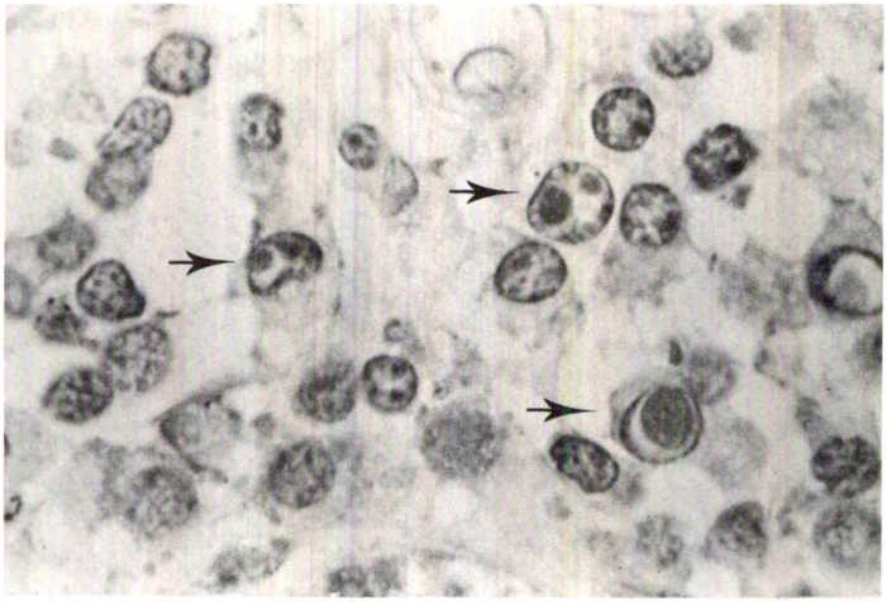

FIGURE 4. Lymphoplasmacytoid immunocytoma (patient No. 9). Note intranuclear PAS-positive globular inclusions (arrows), (× 1400).

Immunohistochemistry and immunofluorescence of the neoplastic cells

Immunologically the neoplastic cells in two patients with centroblastic lymphoma (Nos 1 and 2) were characterized by a monoclonal staining pattern for SIg, a weak staining for CIg of the same heavy and light chain class, the presence of C3-receptors and the absence of HLA-DR (Ia-like) antigens (*see* Table 3). In contrast, the neoplastic cells in cases 3 and 4 strongly expressed Ia-like antigens, stained for SIg, but remained completely negative when stained for CIg. From the two patients with centroblastic/centrocytic lymphoma a monoclonal staining pattern for SIgM and kappa light chains and negative staining for CIg was observed in one of them (No. 6), whereas staining for both SIg and CIg remained completely negative in patient No. 5. In both cases the neoplastic cells had C3-receptors and expressed HLA-DR antigens. By immunofluorescence examination in the five patients with immunocytoma a large proportion of the neoplastic cells showed a monoclonal staining pattern for SIg and CIg (Table 3). Immunoperoxidase studies on paraffin-embedded material demonstrated large accumulations of cells containing CIg of the same heavy and light chain class as the SIg (Fig. 5). At the periphery of the infiltrates, often there were reactive polyclonal plasma cells which stained much more intensely for CIg and J chain as compared to the neoplastic cells. Cells showing adherence with EAC-complexes were only found in those areas containing small cleaved cells (centrocytes) and were not detected in areas predominantly composed of lymphoplasmacytoid and plasma cells. The histological and immunological data of the malignant lymphoma of the ethmoid, which had been present in case 11 six years prior to this study, were very similar to those of the present skin biopsy sections, showing a monoclonal staining pattern for intracytoplasmic IgM and kappa light chains.

Enzyme histochemistry

The majority of the neoplastic cells of the patients with centroblastic and centroblastic/centrocytic lymphoma showed membrane-associated 5-nucleotidase (Nos 1, 2, 3 and 6) and/or

TABLE 3. Characteristics of the neoplastic B cells and modulatory T cells in the dermal infiltrates of eleven patients with a cutaneous B-cell lymphoma

		Neoplastic cells						Modulatory T cells			
Patient	Classification	SIg/ CIg	J chains	C3	BA-1	BA-2	HLA-DR	Leu-1	Leu-2a/ OKT8	Leu-3a/ OKT4	TA-1
1	Centroblastic	$\mu\lambda$	—	+ + +	—	—	—	±	−/±	±	±
2	Centroblastic	$\gamma\kappa$	ND	+ + + +	—	+ +	—	±	−/±	±	±
3	Centroblastic	$\gamma\kappa$	ND	+ + + +	+ + + +	+ + + +	+ + + +	±	−/±	±	±
4	Centroblastic	$\mu\kappa$	—	+ + +	—	+ + +	+ + + +	+	±	+	+
5	Centroblastic/ centrocytic	—	—	+ + +	—	—	+ + + +	+ + +	+	+ + +	+ + +
6	Centroblastic/ centrocytic	$\mu\kappa$	+	+ + +	ND	ND	+ + + +	+ + +	+	+ + +	+ + +
7	Immunocytoma, lymphoplasmacytic	$\mu\kappa$	—	—	—	—	+	+ +	+	+ +	+ +
8	Immunocytoma, lymphoplasmacytic	$\mu\lambda$	+ +	—	ND	ND	ND	ND	ND	ND	ND
9	Immunocytoma, lymphoplasmacytoid	$\mu\lambda$	—	+	+ + +	+	+ +	+ + +	+	+ + +	+ + +
10	Immunocytoma, polymorphic	$\mu\kappa$	+ +	±	—	+ +	+	+ + +	+	+ + +	+ + +
11	Immunocytoma, polymorphic	$\mu\kappa$	±	+	+ + +	+ +	+ +	+ + +	+ +	+ + +	+ + +

SIg = surface immunoglobulins; CIg = intracytoplasmic immunoglobulins; C3 = receptor for the activated third component of complement.

± = 1–5%, + = 6–15%, + + = 16–30%, + + + = 31–60%, + + + + = more than 60% of the cells.

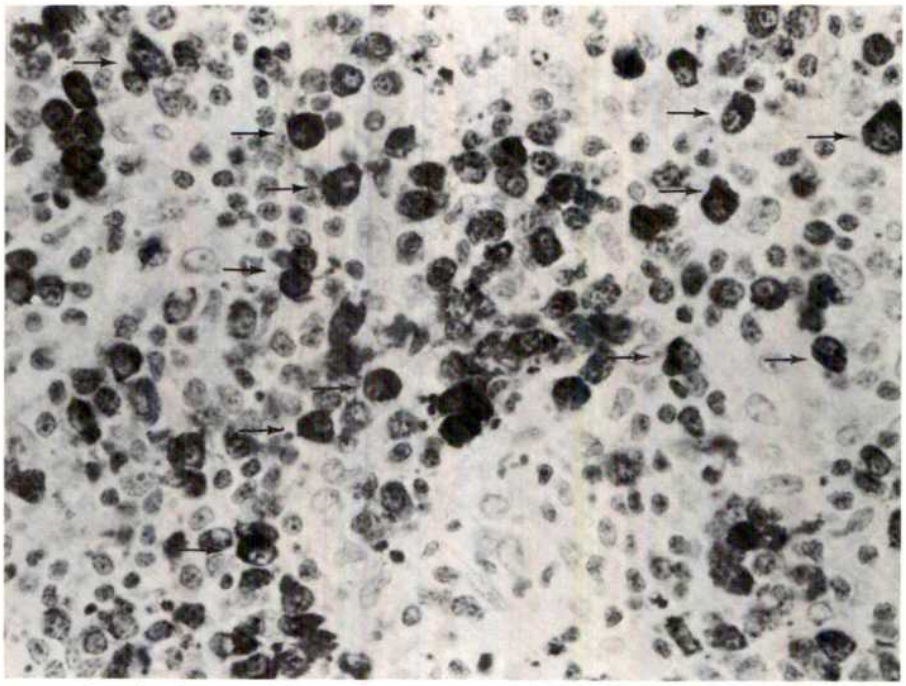

FIGURE 5. Lymphoplasmacytoid immunocytomas (patient No. 9). Many cells show intracytoplasmic staining for lambda light chains (arrows), (× 550).

ATP-ase (Nos 1, 2, 4, 5 and 6) activity. In the dermal infiltrates of the patients with immunocytoma approximately 50 per cent of the cells stained positively with these enzymes. In case 9 ATP-ase activity was clearly restricted to the lymphoplasmacytoid and plasma cells at the periphery of the infiltrates, whereas the central areas containing many centrocytes stained positively for 5-nucleotidase and also for alkaline phosphatase. In addition staining for alkaline phosphatase was strongly positive in one patient with centroblastic lymphoma (No. 3), but completely negative in all other cases. The lymphoplasmacytoid and plasma cells in all patients with immunocytoma showed strongly granular acid phosphatase activity, which was often accumulated at one side of the cytoplasm.

Many macrophages which were characterized by a strong and diffuse staining for ANAE and acid phosphatase, were found diffusely scattered amongst the neoplastic cells in most cases. Remarkably, in the two cases with the lymphoplasmacytic subtype of immunocytoma, in which the dermal infiltrates were mainly composed of cells showing a granular acid phosphatase activity, the number of macrophages was relatively low. Dendritic reticulum cells characterized by their elongated nuclei and long cytoplasmic projections showing ANAE but no acid phosphatase activity, were numerous in centroblastic/centrocytic lymphoma and in the areas containing centrocytes in some immunocytomas (Fig. 6). In centroblastic lymphoma relatively few dendritic reticulum cells were observed.

Monoclonal antibodies

The two monoclonal antibodies reactive with all peripheral T cells, anti-Leu-1 and TA-1, produced patterns that were very similar to one another. In centroblastic lymphoma very few

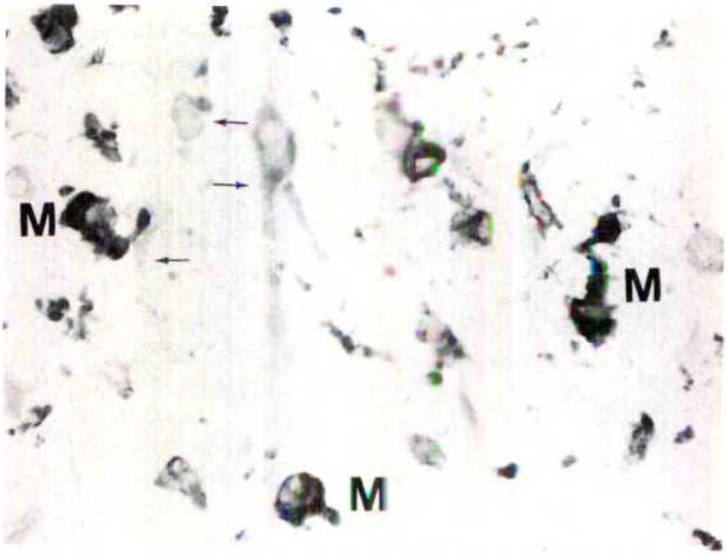

FIGURE 6. Polymorphic subtype of immunocytoma (patient No. 10). Alpha-naphthyl acetate esterase staining, showing dendritic reticulum cells (arrows) and macrophages (M). (× 640).

positive cells were found scattered amongst the neoplastic cells, whereas in centroblastic/centrocytic lymphoma approximately 30–50% of the cells expressed these antigens. In the four cases with immunocytoma, large areas were observed in which the majority of the cells expressed Leu-1 and TA-1 antigens, whereas in other parts of the infiltrate only a small proportion of the cells was found to be reactive with these antibodies (Fig. 8). The overall percentage of Leu-1 and TA-1 positive cells was estimated to be 20% in case 7 and 40–50% in the other cases. In all ten cases studied the percentages of Leu-2a$^+$ and Leu-3a$^+$ cells were identical to the percentages of OKT8$^+$ and OKT4$^+$ cells respectively, suggesting that these antisera define closely related antigens. The distribution of Leu-3a$^+$, OKT4$^+$ cells (helper/inducer T-cell phenotype) and Leu-2a$^+$, OKT8$^+$ cells (cytotoxic/suppressor T cell phenotype), differed considerably. Whereas Leu-3a$^+$, OKT4$^+$ cells tended to form large clusters, Leu-2a$^+$, OKT8$^+$ cells were diffusely scattered or localized at the periphery of the infiltrates (Fig. 7). The OKT4, Leu-3a/OKT8, Leu-2a ratios varied between 5:1 and 2:1.

The percentages of cells reactive with anti-HLA-DR antibody in two patients with centroblastic/centrocytic lymphoma ranged from 60–80%, indicating that not only the neoplastic B cells, but also the T cells in these infiltrates expressed Ia-like antigens. In the patients with immunocytoma the percentages of HLA-DR$^+$ cells were generally low, except for case 9. In this patient many centrocytes (approximately 30% of the cells) expressed HLA-DR antigens. Staining with OKT6 and NA 1/34 antisera showed dendritic cells in the suprabasal layers of the epidermis, which probably represent Langerhans cells and/or related cells. Identical cells were occasionally observed in the grenz zone between the epidermis and the dermal infiltrate.

Varying numbers of lymphoid cells stained for BA-1 and BA-2 antisera. However, no uniform pattern was observed (see Table 3). In addition, BA-2 antiserum stained large, dendritic, non-lymphoid cells which morphologically resembled macrophages and/or dendritic reticulum cells.

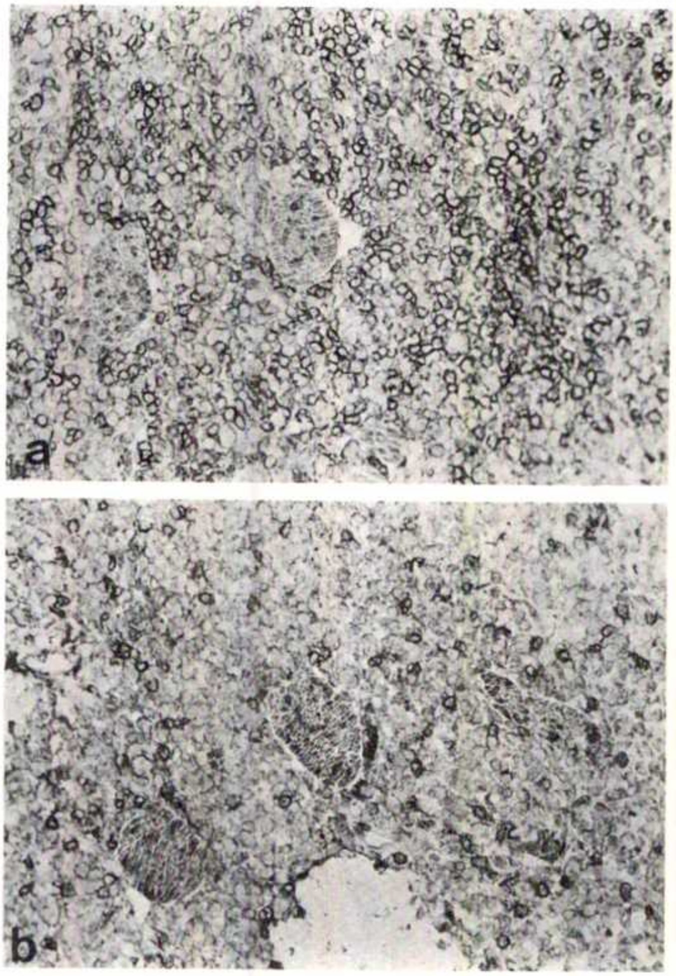

FIGURE 7. Distribution of T-cell subsets in centroblastic/centrocytic lymphoma (patient No. 5), showing (a) many Leu-3a$^+$ T cells and (b) few, diffusely scattered Leu-2a$^+$ T cells.

DISCUSSION

In the present study the cellular composition of the dermal infiltrates of eleven patients with cutaneous B-cell lymphoma was investigated. This group included four immunoblastic lymphomas, two centroblastic/centrocytic lymphomas and five immunocytomas (Keil classification; Gérard-Marchant *et al.*, 1974; Lennert *et al.*, 1975). Cutaneous involvement was the presenting symptom in ten patients. In eight of them the malignant lymphoma apparently remained confined to the skin for at least 6 months, suggesting a primary cutaneous origin. A monoclonal staining pattern for SIg and/or CIg was found in all cases but one (Case No. 5). In this patient with centroblastic/centrocytic lymphoma, which showed a negative staining for both SIg and CIg, the diagnosis of B-cell lymphoma was based on the characteristic histological picture, the presence of C3-receptors and Ia-like antigens, and the absence of T-cell and histiocytic markers (absence of Fc γ-receptors, negative staining for lysozyme, alpha$_1$-antitrypsin, alpha$_1$-antichymotrypsin, acid phosphatase and ANAE). Whereas benign centroblasts

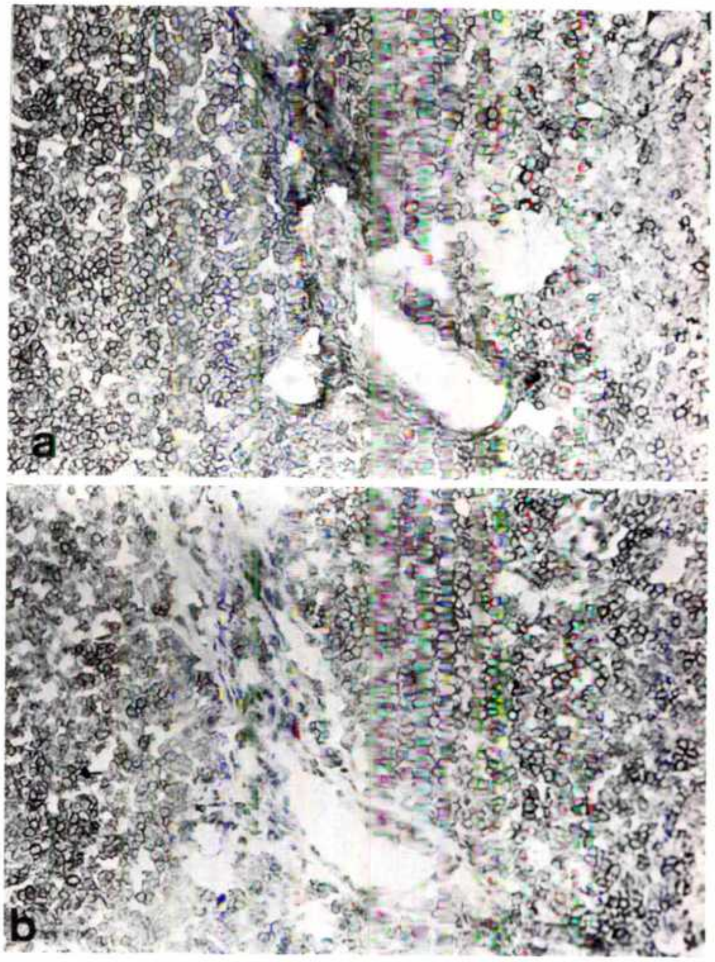

FIGURE 8. Distribution of neoplastic B cells (a) and modulatory T cells (b) in lymphoplasmacytoid immunocytoma (patient No. 9). Note central blood vessel. At the left side a predominance of SIgM$^+$ B cells and few Leu-1$^+$ T cells, at the right side a predominance of Leu-1$^+$ T cells and relatively few SIgM$^+$ B cells.

normally show an intense staining for Ia-like antigens (Stein *et al.*, 1980), the neoplastic cells in two out of four patients with centroblastic lymphoma were completely negative when stained with anti-HLA-DR antiserum. In both cases only scattered macrophages were found to be reactive with this antiserum.

With respect to the distribution of T-cell subpopulations, dendritic reticulum cells and macrophages in these cutaneous B-cell lymphomas, the dermal infiltrates of the patients with centroblastic lymphoma showed only occasional T cells, few dendritic reticulum cells (as demonstrated by a dendritic ANAE activity), and relatively many macrophages. These results are in agreement with the findings in centroblastic lymphomas in lymph nodes (Lennert *et al.*, 1978; Stein *et al.*, 1981). Moreover, a similar distribution of modulatory T cells, dendritic reticulum cells and macrophages was found in an axillary lymph node recently obtained from patient No. 3, which also showed centroblastic lymphoma. The immunological and cytochemical characteristics of the neoplastic cells in this lymph node were identical to those in the skin.

In the two cases with centroblastic/centrocytic lymphoma numerous dendritic reticulum cells and macrophages and a considerable number of Leu-1$^+$, TA-1$^+$ T cells were found interspersed amongst the neoplastic cells. The majority of these T cells was also reactive with anti-Leu-3a and OKT4 antisera, which define an inducer/helper T-cell subpopulation, whereas a relatively small percentage expressed Leu-2a and OKT8 antigens (cytotoxic/suppressor T-cell phenotype). Although no double-labelling studies were performed, the very high percentages of HLA-DR$^+$ cells indicate that the majority of these T cells also express Ia-like antigens and represent activated T cells (Reinherz *et al.*, 1979c; Yu *et al.*, 1980). The presence of numerous dendritic reticulum cells, macrophages and activated OKT4$^+$ cells, which has also been described in secondary follicles and in follicular centroblastic/centrocytic lymphoma in lymph nodes (Stein *et al.*, 1980, 1982) suggests persistent stimulation of the whole follicular compartment. Persistent antigenic stimulation and loss of normal regulatory influences have been suggested to be responsible for the development of frank malignancy (Bérard *et al.*, 1981).

In the five cases with immunocytoma studied, dendritic reticulum cells, as demonstrated by a dendritic ANAE activity, were relatively few and preferentially located in those areas which also contained centrocytes and centroblasts. The percentages of cells reactive with the anti-Leu-1 and anti-TA-1 pan-T cell antisera ranged from 20–50%. The predominance and the distribution pattern of Leu-3a$^+$, OKT4$^+$ T-helper cells is consistent with our findings in immunocytomas in lymph nodes (Meijer, Willemze & van der Valk, in preparation). No differences were found between primary and secondary cutaneous immunocytomas. The absence of activated T cells and the relatively low number of dendritic reticulum cells in these immunocytomas contrasts with the findings in centroblastic/centrocytic lymphoma and suggests different pathogenetic mechanisms involved in the development of these different types of B-cell lymphoma. Moreover, in contrast to centroblastic/centrocytic lymphomas, immunocytomas rather frequently show primary cutaneous involvement (Kerl *et al.*, 1982) and can be limited to the skin for a long period of time (Guggenberger *et al.*, 1982).

In summary, it is generally accepted that B-cell lymphomas in lymph nodes are the neoplastic counterparts of B-cell reactions which usually take place after antigenic stimulation in the corresponding lymph node compartments. Our findings that not only the neoplastic cells, but also the micro-environment, i.e. the distribution of reactive T-cells, dendritic reticulum cells and macrophages in primary cutaneous B-cell lymphomas is very similar to that in secondary cutaneous B-cell lymphomas and B-cell lymphomas of the same type in lymph nodes, suggest that these primary cutaneous B-cell lymphomas are the neoplastic counterparts of B-cell reactions which usually take place in the skin after antigenic stimulation, and which are closely related to similar reactions in the lymph node.

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Quantitative analysis of T-lymphocyte subsets in atopic eczema, using monoclonal antibodies and flow cytofluorimetry

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SUMMARY

An analysis of T-lymphocyte subpopulations was performed on thirty-eight consecutive patients with atopic eczema, using monoclonal antibodies and flow cytofluorimetry. The total T-lymphocyte and helper T-cell numbers did not differ significantly from those of a matched control group, but there was a highly significant reduction in suppressor T-cell numbers among the atopic group. Both suppressor T-cell deficiency and circulating IgE elevation were found to correlate positively with disease activity.

Food specific IgE levels to milk and egg were ascertained by radioallergosorbent (RAST) studies. These proved positive in seven out of thirty-five atopics (20%).

Atopic eczema is associated with impaired cell-mediated immunity, as shown by increased susceptibility to bacterial, viral and fungal infection, relative anergy to PPD and reduced contact sensitivity (Hanifin & Lobitz, 1977).

Imbalances of T-lymphocyte subpopulations may play an important role in the pathogenesis of atopic eczema (Schuster *et al.*, 1980; Stingl *et al.*, 1981; Rola-Pleszczynski & Blanchard, 1981). In particular a deficiency of suppressor T cells may result in increased IgE production following challenge with common environmental allergens. In the rat, IgE production is known to be subject to suppressor T-lymphocyte control and IgE responses can be increased by therapy which predominantly inhibits suppressor T-cell function (Okumara & Tada, 1971; Taniguchi & Tada, 1971; Tada, Taniguchi & Okumara, 1971). Human *in vitro* studies have demonstrated both inhibition of IgE synthesis by suppressor T cells (Gehr *et al.*, 1980) and inhibition of elevated IgE synthesis from atopic lymphocytes after coculture with normal T lymphocytes (Fiser & Buckley, 1979; Saxon, Morrow & Stevens, 1980).

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A positive correlation between disease activity and suppressor T-cell deficiency has been reported (Canonica *et al.*, 1979; Schuster *et al.*, 1980). However, Stingl (1981) found reduction of suppressor T-cells numbers to be very consistent and independent of the severity of involvement. Several recent *in vivo* studies of atopic eczema have shown an inverse relationship between IgE levels and suppressor T-cell activity (Byrom *et al.*, 1979; Schuster *et al.*, 1980; Rola-Pleszczynski & Blanchard, 1981), although this has not been confirmed by others (Canonica *et al.*, 1979; Leung, Rhodes & Gehr, 1981). IgE elevation is presumably related to reduced suppression of B-lymphocyte response to common antigenic stimuli.

Previous assessment of total T-lymphocyte counts in atopic eczema has yielded conflicting results (Byrom *et al.*, 1979; Gottlieb & Hanifin, 1974; Luckasen *et al.*, 1974; McGeady & Buckley, 1975; Grove, Reid & Forbes, 1975; Carapeto, Winkelmann & Jordon, 1976; Secher, Svejgaard & Sonderstrup-Hansen, 1977; Hovmark, 1977; Saraclar McGeady & Mansmann, 1977; Stingl *et al.*, 1981). Nevertheless several groups have observed a diminished population of circulating suppressor T cells based on estimation of the numbers of lymphocytes bearing Fc IgG receptors (Moretta *et al.*, 1977; Pichler, Lum & Broder, 1978; Schuster *et al.*, 1980; Stingl *et al.*, 1981).

Recently it has become possible to identify specific T-cell subpopulations using monoclonal antibodies (Milstein *et al.*, 1979); Kung *et al.*, 1979; Reinherz *et al.*, 1980; Hoffman *et al.*, 1980). In addition the use of flow cytofluorimetry provides an objective, reproducible and accurate computerized estimation of cell numbers by identifying individual fluorochrome labelled cells as they pass through a laser source (Hoffman *et al.*, 1980).

We have used monoclonal antibodies and flow cytofluorimetry to quantify circulating T-lymphocyte subpopulations in atopic eczema. The results obtained have been correlated with age, disease activity, total circulating IgE levels and food specific IgE levels as measured by the radioallergosorbent (RAST) test.

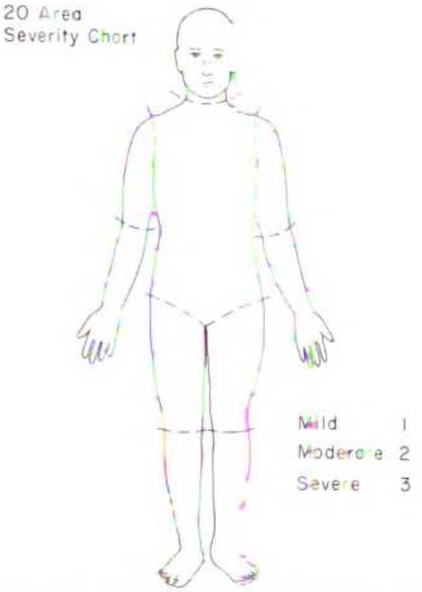

FIGURE 1. Each of twenty areas was subjectively assessed and scored from 1 to 3 according to mild, moderate or severe disease activity.

PATIENTS

T-lymphocyte subpopulation studies were performed on thirty-eight consecutive patients aged between 10 and 45 years (average age 22 years) suffering from active atopic eczema (AE). The diagnosis was established by standard clinical criteria including physical examination, a positive personal and/or family history of atopy, elevated serum IgE concentration and histopathological features. The extent of skin involvement was defined by use of a 'twenty area' severity chart (Fig. 1), subjectively measuring the severity of eczema in each area by a 1–3 grading (indicating mild, moderate or severe activity) to give a total score of 0–60. Patients with infection of the skin or previous systemic steroid therapy were excluded. Topical steroid therapy and oral antihistamines were withdrawn at least 72 h prior to venesection. Twenty-three healthy non-atopic controls aged between 19 and 54 years (average age: 29 years) were also studied.

METHODS

Peripheral blood mononuclear cells were obtained from 5 ml of heparinized blood layered onto an equal volume of Ficoll–Paque (Pharmacia) and centrifuged at 2000 rpm for 20 min at 18°C. The lymphocyte-rich buffy coat was drawn off and washed twice in PBS before resuspending the cells at a concentration of 1×10^6 in PBS supplemented with 5% fetal calf serum and 0·3% sodium azide. These conditions provided a stable environment for the lymphocytes while restricting cellular metabolism.

The Orthoclone series of monoclonal antibodies, OKT3, OKT4 and OKT8 identify respectively all mature peripheral blood T lymphocytes, helper T cells (comprising approximately 65% of peripheral blood T lymphocytes) and suppressor T cells, comprising about 35% of the peripheral blood T-lymphocyte population (Reinherz *et al.*, 1979; Reinherz *et al.*, 1980). ICHT1 is a pan T-cell marker produced and characterized by the ICRF Human Tumour Immunology Group, University College Hospital Medical School, London (Beverley & Callard, 1981).

One hundred μl aliquots of mononuclear cell suspensions were incubated with each of the monoclonal antibodies in turn at a dilution of 1:40 for 30 min and agitated manually every 10 min. After washing the cells twice in PBS, secondary incubation was performed with fluorescein-conjugated rabbit anti-mouse immunoglobulin antibody (DAKO) at a dilution of 1 in 100 for 30 min. Non-specific fluorochrome labelled antibody binding to lymphocyte surface Fc receptors was abolished by absorption through a human IgG column.

Background staining was determined by incubation of the mononuclear cell suspension with the secondary antibody alone. Immunoglobulin capping on the lymphocyte surface was prevented by maintaining the cells at 4°C and by the use of 0·3% sodium azide throughout the whole procedure.

Subsequent fixation with 1% paraformaldehyde for 20 min allowed quantitative analysis by flow cytofluorimetry(FACS) to be performed up to 2 weeks later, assessing at least 10,000 cells per sample.

Total IgE estimation was performed on each atopic and control subject using a liquid phase double antbody technique. The normal range was below 135 iu/ml.

Food specific IgE antibodies against egg and milk were also estimated using purified antigens in the radioallergosorbent (RAST) test (Johansson, 1975).

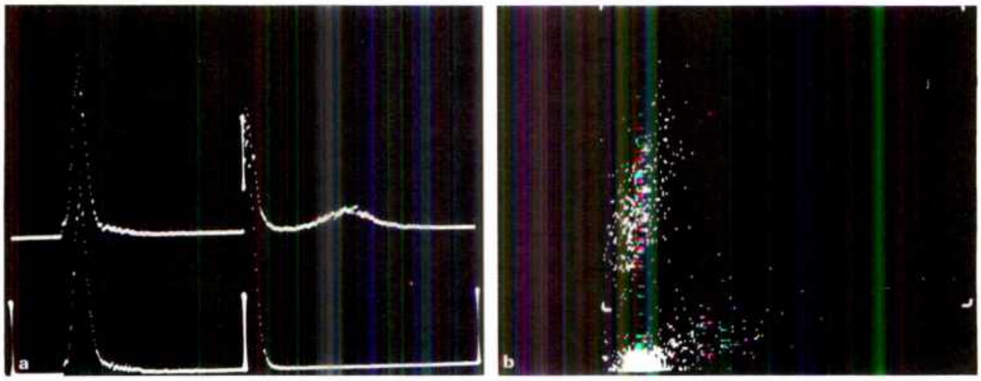

FIGURE 2. (a) FACS 4 histogram displaying cell size and fluorescence, both with (above) and without (below) primary incubation with monoclonal antibody. The presence of a discrete peak of fluorescence is noted at the upper level indicating reactivity with OKT4. (b) FACS 4 cytogram demonstrating T-cell reactivity with monoclonal antibody (OKT4). Each dot represents one cell assessed for size (x axis) and fluorescence (y axis). There is clear separation of fluorescent helper T cells (above) from background fluorescence (below).

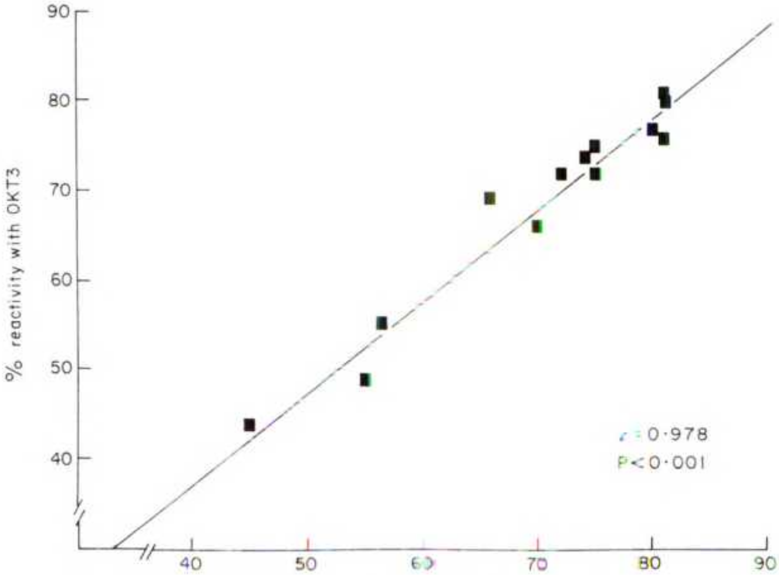

FIGURE 3. UCHT1 and OKT3 identify the total T-cell population, and a high degree of correlation was confirmed.

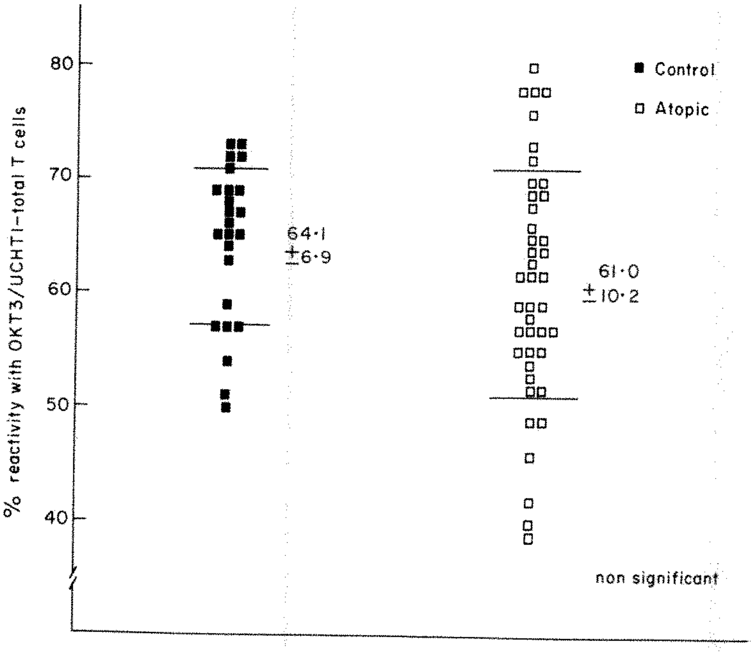

FIGURE 4. No significant difference was found between the total T-lymphocyte numbers in the AE and control groups.

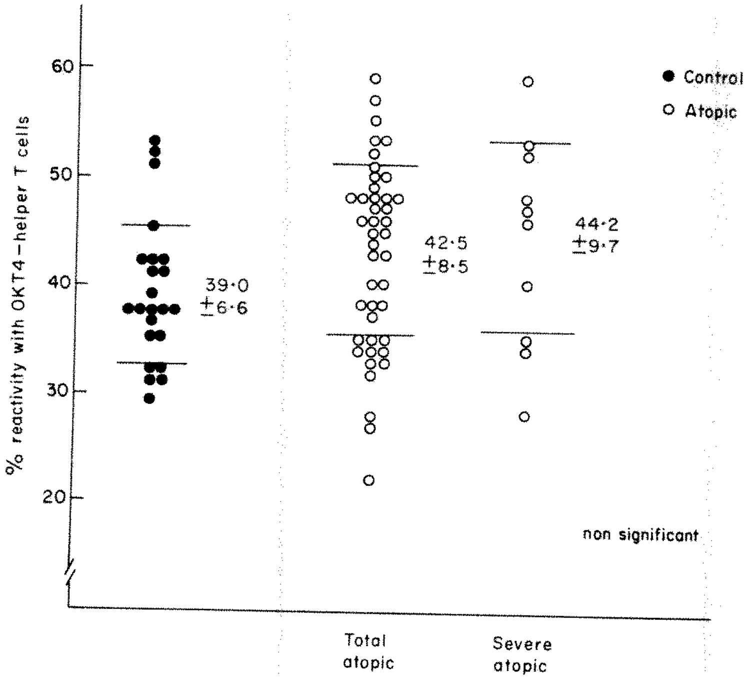

FIGURE 5. There was a trend towards increased numbers of helper T cells in AE. However, this failed to reach statistical significance.

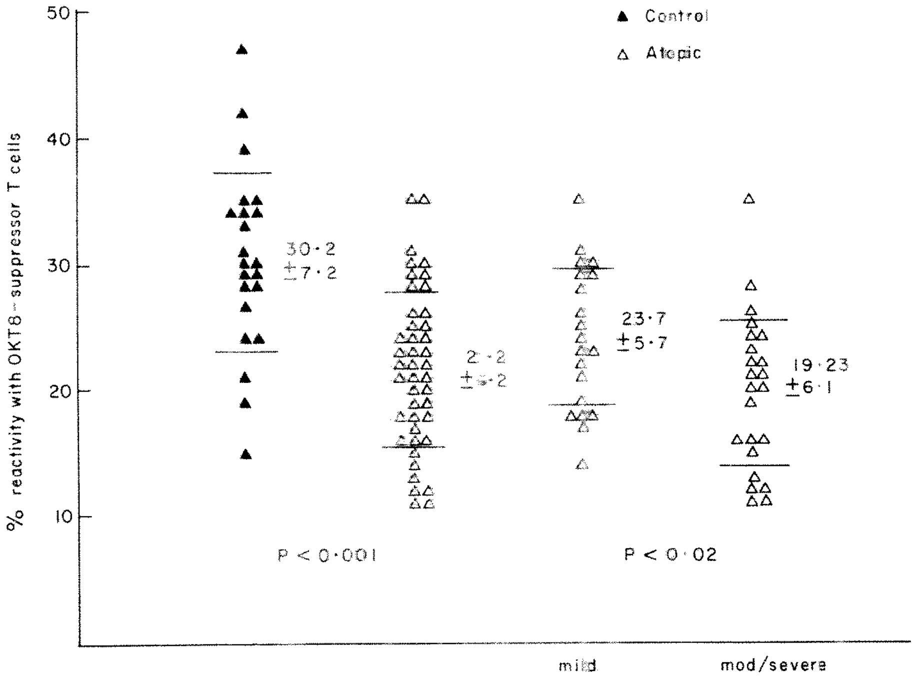

FIGURE 6. A highly significant reduction in AE suppressor T-cell numbers was accentuated by comparison of mild with moderate and severe groups.

Statistical methods

All results were analysed for normality using the probability plot correlation coefficient test—Fillibens test (Filliben, 1975). The presence of outliers was ascertained by Grubbs T test. Those results which were normally distributed were subjected to a two-tailed Student's t test, and non-parametric results to the Mann–Whitney U test. The T-cell subset findings are expressed as a percentage of peripheral blood lymphocytes giving the mean value ± one standard deviation.

RESULTS

Cell size and fluorescence were instantly viewed in either histogram (Fig. 2a) or cytogram (Fig. 2b) form. In each analysis at least 10,000 cells were individually assessed, with a turnover of one sample per min. The fluorescence relating to T-lymphocyte reactivity with monoclonal antibody is clearly demonstrated and well separated from the background fluorescence.

Reactivity of T cells with UCHT1 and OKT3 was found to be almost identical (Fig. 3). The normal total T-lymphocyte population for control subjects, as measured by combined OKT3/UCHT1 positivity, was 64·4% ± 6·9% of circulating lymphocytes. By comparison the atopic subjects were found to have a total T-cell population of 61% ± 10·2% (Fig. 4). The difference was not statistically significant.

The helper T-cell population as indicated by OKT4 reactivity was found to be 39% ± 6·6% in normal controls and 42·5% ± 8·5% in the atopic group. Again there was no statistical difference between these figures but the upward trend was emphasized when comparison was made between the total atopic and severe atopic groups (Fig. 5).

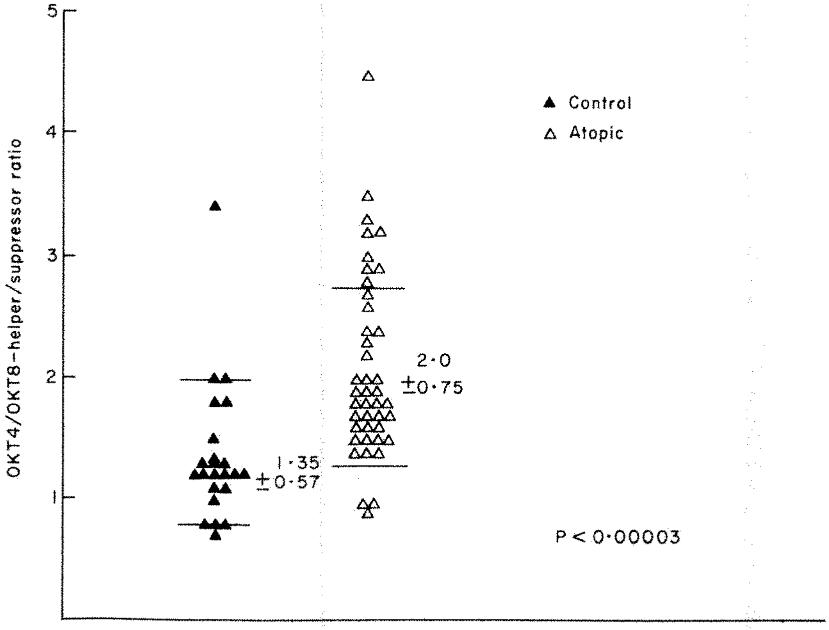

FIGURE 7. Helper/suppressor ratios were found to be significantly elevated in the AE group ($P < 0{\cdot}00003$, Mann–Whitney U test).

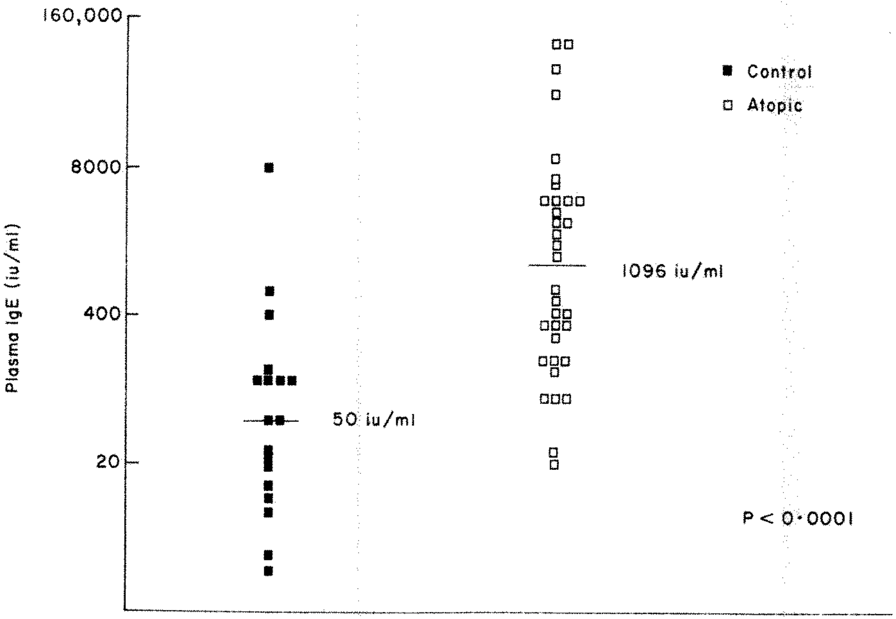

FIGURE 8. Total serum IgE concentrations showed a wide variation amongst AE subjects, and were significantly elevated in comparison with the control group ($P < 0{\cdot}0001$).

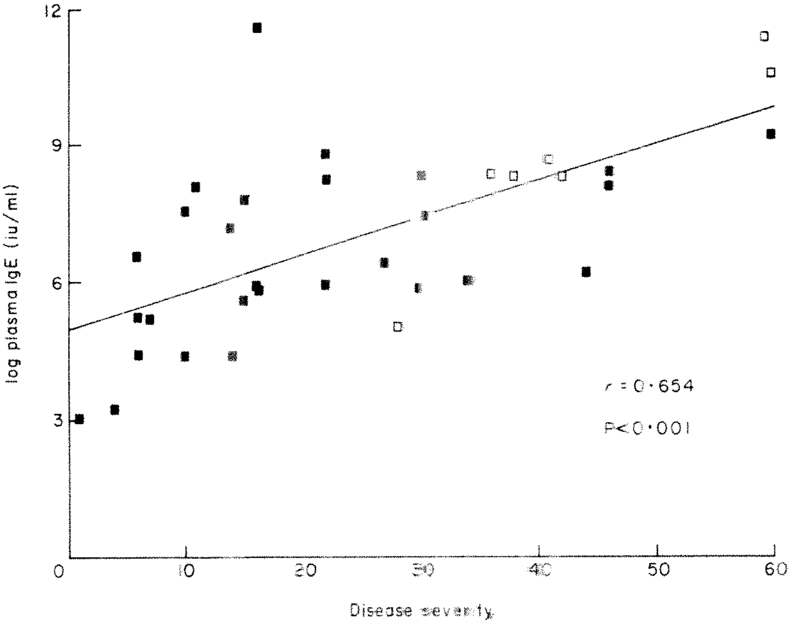

FIGURE 9. Total serum IgE levels correlated positively with disease severity. The unfilled squares represent those patients with positive milk or egg RASTs.

OKT8 positivity (which probably reflects suppressor cell numbers) showed a highly significant difference between the control group (30·2% ± 7·2%) and the atopic group (21.2% ± 6·2%). This difference was again accentuated by further comparison of mild atopic with moderate and severe atopic groups (Fig. 6). Suppressor T-cell deficiency correlated positively but weakly with disease activity ($r = 0{\cdot}367$).

All the above data were normally distributed. However, Fillibens test for the assessment of normality of OKT4/OKT8 ratios demonstrated a non-parametric distribution. The mean control ratio of 1·35 ± 0·57 differed very significantly from the atopic ratio of 2·0 ± 0·75. (Mann Whitney U test, $P < 0{\cdot}00003$), (Fig. 7).

When expressed logarithmically the total IgE levels assumed a normal distribution. Three out of twenty-three control subjects had elevated total IgE values including one at 8800 units. Conversely, six out of thirty-four (18%) atopic patients had IgE levels within the normal range. Comparison of the medians showed an obvious elevation of IgE levels in the atopic group which was highly significant (Fig. 8). Total IgE levels correlated positively with disease severity ($r = 0{\cdot}654$) (Fig. 9), but surprisingly not with suppressor cell numbers. This may be explained by the weak correlation of OKT8 positivity with disease severity.

Milk and egg RAST studies on thirty-five atopic eczema patients proved positive in seven (20%), of which six had either moderate or severe disease. However, five of these were unable to give a good history of food-related exacerbation. One of twenty-three control subjects (4%) was found to exhibit a weakly positive RAST to milk.

DISCUSSION

Development of specific monoclonal antibodies against human T-lymphocyte subpopulation antigens (Reinherz *et al.*, 1980) has provided an accurate and reproducible method for the analysis of T-cell subsets which possesses several technical advantages over the methods previously employed. The ability of T lymphocytes to form E rosettes with sheep red blood cells has been variously reported as being reduced or normal in patients with atopic eczema (Stingl *et al.*, 1981) Unfortunately not all E rosetting (E+) cells are T cells. Indeed it appears that there is a subpopulation of E+ cells without other T-cell markers or evidence of mature T-cell function (Beverley, 1981). Furthermore E rosetting techniques do not provide any information concerning T-cell subsets. Fc IgG bearing cell numbers (Tγ) denoting suppressor T-cell populations have generally been found to be reduced while Fc IgM bearing cells (Tμ) indicating helper cells were found in normal concentrations (Moretta *et al.*, 1977; Schuster *et al.*, 1979; Stingl *et al.*, 1981). Reduction in suppressor cell function has also been demonstrated by concanavalin A (Con A) inducible suppressor cell studies (Rola-Pleszczynski & Blanchard, 1981; Stingl *et al.*, 1981). These indirect methods are relatively complex and time consuming. Conversely the use of monoclonal antibodies coupled with flow cytofluorimetry allows simple, precise and rapid identification of T cells and their subsets.

Our results confirm a deficiency of suppressor T-lymphocyte numbers with a direct relationship to disease activity. Of the five patients studied both before and after treatment the helper/suppressor ratio was reduced in three and unchanged in two. This may represent a normalizing effect of treatment or clinical improvement on T-cell subset abnormalities.

It has previously been demonstrated that the ability to generate Con A induced suppressor T cells is dependent on disease activity (Stingl *et al.*, 1981). Desensitization of patients with atopic eczema in association with clinical improvement leads to correction of abnormalities of circulating T-cell numbers (Canonica *et al.*, 1981). Rocklin (1981) showed that antigen specific suppressor T-cell activity improved after successful desensitization for ragweed allergy. These findings suggest that the impaired production of suppressor cells in clinically active atopic eczema is a secondary phenomenon.

Contrary evidence indicating a primary role for suppressor T-cell deficiency in the development of atopic eczema is suggested by prospective studies on 1-month-old babies. Infants who were found to have significantly lower T-cell numbers than the control children subsequently developed atopic eczema (Juto & Strannegard, 1979). Earlier antigen exposure of genetically susceptible children may explain these findings.

Increased IgE levels commonly associated with atopic eczema may be directly related to both increased helper activity as well as reduced suppressor activity. However we have not been able to demonstrate a direct relationship between T-cell subsets and the total circulating IgE levels. Considerable significance has been placed on total serum IgE levels in the past. However, 18% of our atopic eczema patients had normal IgE levels. Brostoff, Johns & Stanworth (1977) stressed the importance of complexed IgE in atopic eczema. High molecular weight IgE polymer was demonstrated in fifteen out of sixteen atopic patients amongst whom three had normal levels of total circulating IgE. It is further suggested that IgE complexes may be responsible for the 'late' responses seen in immediate hypersensitivity (Brostoff *et al.*, 1979; Paganelli *et al.*, 1979).

In addition, IgE-carrying peripheral blood lymphocytes are present in increased concentration in atopic eczema (Lobitz, Honeyman & Winkler, 1972; Cormane, 1974; Carapeto *et al.*, 1976). Direct immunofluorescence studies on lesional skin from atopic eczema had demonstrated lymphoid cells with membrane-bound IgE, particularly in and around blood vessels and scattered throughout the dermis, and also around eczematous vesicles in the epidermis (Cormane, Husz & Hamerlinck, 1974).

Circulating IgG4 is reported to be increased in atopic eczema patients (Shakib *et al.*, 1977; Gwynn *et al.*, 1979) and a positive correlation has been found between IgG4 and IgE antibodies in atopic diseases (Bruynzeel & Berrens, 1979). In anaphylactic hypersensitivity IgG4 is a significant antibody to some antigens (Parish, 198[illegible]).

The high numbers of atopic eczema patients with food positive RASTs supports the suggestion of a dietary factor in the aetiology of this condition and should be considered in its clinical management.

Defective suppressor T-cell activity in atopic eczema may affect total circulating IgE levels, circulating complexed IgE, or local production of IgE in tissue. Alternatively, an effect on the circulating and tissue IgG4 concentrations may be of relevance. Under normal circumstances suppressor T cells have been found to exert a controlling influence on cytotoxic activity of lymphocytes against autologous skin fibroblasts (Parkman & Rosen, 1976; Osband & Parkman, 1978). The reduction of suppressor T cells in atopic dermatitis might result in an immune-mediated skin injury by naturally occurring auto-cytotoxic lymphocytes (Leung, Rhodes & Geha, 1981). Further investigations of all these aspects is warranted while the precise relationship between suppressor T-lymphocyte deficiency and atopic eczema remains unclear.

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In situ identification of T6-positive cells in normal human dermis by immunoelectron microscopy*

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SUMMARY

Monoclonal anti-T6 antibody, which reacts with the majority of cortical thymocytes but not peripheral T cells, also reacts with human epidermal Langerhans cells, as shown by a four-step immunoperoxidase method and immunoelectron microscopy. To define whether T6-positive cells are also present in normal human dermis, we used these techniques to demonstrate two immunologically distinct populations of histiocyte-like cells in normal human dermis. The first population contains cells devoid of phagolysosomes or Birbeck granules. These cells react with anti-T6 antibody, but not with monoclonal anti-T3 antibody which defines peripheral T cells, and are found predominantly in and around dermal lymphatic vessels. The second is composed of phagolysosome-containing cells which do not react with anti-T6 antibody or anti-T3 antibody.

Because to date, Langerhans cells are the only cells in normal human epidermis that react with anti-T6 antibody, these data provide immunological evidence for a specific link between Langerhans cells and a T6-positive dermal mononuclear cell, possibly the so-called indeterminate cell. In addition, application of these techniques should, for the first time, permit the immunological distinction of these T6-positive mononuclear cells from other cells bearing Ia antigens, such as dermal histiocytes and certain lymphocytes, in normal and diseased skin.

Langerhans cells are important in the induction of cutaneous contact hypersensitivity (Silberberg-Sinakin & Thorbecke, 1980) and potentially in the recognition and processing of tumour-associated antigens (Kripke, 1980). Normal epidermal Langerhans cells have Fc and

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C3 receptors (Stingl *et al.*, 1977) and express HLA-DR antigens (Ia-like antigens) (Rowden, Lewis & Sullivan, 1977). These cells appear to originate in the bone marrow, but the mechanism whereby they populate and replenish their numbers within the epidermis is poorly understood.

In 1975, Breathnach defined the indeterminate cell as a dendritic nonkeratinocyte without specific features such as Langerhans cell (Birbeck) granules, melanosomes or Merkel cell granules within their cytoplasm. Since that time, indeterminate cells have been compared to histiocytes, transformed lymphocytes, modified melanocytes and Langerhans cells. Because indeterminate cells bear Ia-like antigens, Rowden, Phillips and Lewis have suggested that they may represent a precursor pool of Langerhans cells (1979). Apparent transitional forms between dermal indeterminate cells and Langerhans cells rich in Birbeck granules in the mid to upper layers of the epidermis have provided support for this hypothesis (Breathnach, 1977).

Monoclonal anti-T6 antibody reacts with the majority of cortical thymocytes, but not with peripheral T cells (Bhan *et al.*, 1980). Poppema and Bhan noted in 1980 that dendritic cells in the paracortices of some human lymph nodes were T6-positive (Poppema *et al.*, 1981). Because similar cells in the mid-epidermis of normal human skin were also T6-positive, we applied immunoelectron microscopy to prove that T6-positive cells in human epidermis are Langerhans cells (Murphy *et al.*, 1981a, b). Subsequently, Fithian *et al.* (1981) provided the supporting evidence that Ia-positive cells in normal epidermis also react with anti-T6 antibody. More recent immunoelectron microscopic studies (Chu *et al.*, 1982) have demonstrated similar findings in epidermal cell suspensions. The present study was undertaken to ascertain whether mononuclear cells *in situ* within normal human dermis also react with anti-T6 antibody. The structure and potential relationships of T6-positive dermal mononuclear cells to Langerhans cells and indeterminate cells are discussed.

MATERIALS AND METHODS

Tissue preparation

Punch biopsies (3 mm) of normal adult skin at least 3 mm deep were obtained from the upper extremities of two healthy male volunteers using 1% Xylocaine® anaesthesia without epinephrine. Initial fixation was 4% paraformaldehyde in 0·1 M sodium phosphate buffer (pH 7·4) containing 8% sucrose for 3 h at 4°C (Hata *et al.*, 1981). After subsequent washing in 0·1 M sodium phosphate buffer containing 8% sucrose, specimens were rapidly frozen in OCT compound (Ames Co., Division of Miles Laboratories, Elkhart, IN, U.S.A.) by suspension in liquid nitrogen, cryostat sectioned at 15 μm and mounted on coated glass slides.

In addition, as positive controls for peripheral T-cell antigens, five 4 mm biopsies of histologically-confirmed lichen planus were frozen in liquid nitrogen and processed as subsequently described for light microscopic examination.

Description of antibodies

The monoclonal antibodies, anti-T3 and anti-T6, which have been previously shown to react with T-cell surface antigens (Reinherz *et al.*, 1979, 1981; Reinherz & Schlossman, 1980; Bhan *et al.*, 1980) were employed. These antibodies were obtained as ascitic fluid from mice injected with hybridoma cells. Control ascitic fluid was obtained from mice injected with non-secreting hybridoma cells. Anti-T3 antibody reacts with approximately 100% of peripheral T cells and 10% of thymocytes. Anti-T6 antibody reacts with approximately 70% of thymocytes and Langerhans cells within the epidermis, but not with circulating T cells or monocytes. In addition, monoclonal antibody directed against anti-I1, the nonpolymorphic region of human Ia-like antigens (Reinherz *et al.*, 1979), was also utilized.

Immunoperoxidase procedures

A highly sensitive four-step immunoperoxidase technique using peroxidase-antiperoxidase complex was used. Frozen tissue sections were overlaid with 25 μl of a 1:500 dilution of mouse ascitic fluid containing either monoclonal anti-T3, anti-T6 or anti-I1 antibodies for 60 min. Subsequently, sections were incubated with a 1:40 dilution of rabbit anti-mouse Ig serum (N.L. Cappel Laboratories, Inc., Cochranville, PA, U.S.A.), a 1:40 dilution of swine anti-rabbit Ig antiserum (Dako Immunoglobulins, Copenhagen, Denmark) and a 1:50 dilution of peroxidase rabbit anti-peroxidase reagent (PAP) (Dako) for 20–30 min. Sections were rinsed in phosphate-buffered saline (PBS) between incubations. Further details of this procedure are reported elsewhere (Poppema *et al.*, 1981).

Sections were stained with 3′ 3′diaminobenzidine (DAB) (Sigma, St Louis, MO, U.S.A.) for 30, 60, 90 and 120 s intervals, rinsed in PBS to terminate the staining reaction and examined by light microscopy. Biopsies of normal skin were further processed for immunoelectron microscopy.

Electron microscopy

Stained sections were fixed with 2% glutaraldehyde in PBS for 20 min at room temperature and stored in Karnovsky's II solution (Karnovsky, 1967) overnight at 4°C. Sections were then examined by light microscopy or post-fixed with 2% osmium tetroxide for electron microscopy. After dehydration in graded ethanol solutions, the sections were embedded in an Epon–Araldite mixture by the inverted gelatin capsule method. Ultrathin sections were cut with a Porter Blum MT-2 ultramicrotome and both unstained sections and sections stained with uranyl acetate and lead citrate were examined with a Phillips 301 electron microscope. When cells resembling indeterminate cells were identified, serial sections at approximately 700 Å intervals were performed to exclude the presence of Birbeck granules within the cytoplasm.

RESULTS

Langerhans cells within the epidermis were clearly demonstrated by immunolight microscopy (Fig. 1) and showed uniform deposition of the reaction product of DAB along the cell membranes by immunoelectron microscopy (Fig. 2). All T6-positive cells within the epidermis contained Birbeck granules within the cytoplasm, although Langerhans cells located in the lower third of the epidermis consistently contained fewer granules than those of the middle and upper thirds.

In and about dermal lymphatic vessels, primarily within the papillary dermis, occasional T6-positive cells were observed (Figs 1 and 3). These cells had irregular, infolded nuclei, numerous mitochondria and cytoplasmic microfilaments (Figs 3 and 4). No Birbeck granules or phagolysosomes were identified. Cells with similar ultrastructural features have been described previously as indeterminate cells (Breathnach, 1975; Rowden *et al.*, 1979). The DAB reaction product was confined to the cell membrane and was less intense than that observed on many Langerhans cells within the epidermis (Fig. 4). All T6-positive cells within the dermis and epidermis were weakly positive when stained by with anti-I1 antibody, and did not stain with anti-T3 antibody.

A second population of mononuclear cells was identified within the papillary dermis, principally disposed about lymphatic vessels and venules. These cells contained phagolysosomes, demonstrated ultrastructural characteristics of conventional histiocytes, and did not react with anti-T6 antibody (Fig. 5). Cells with these features were weakly positive when stained

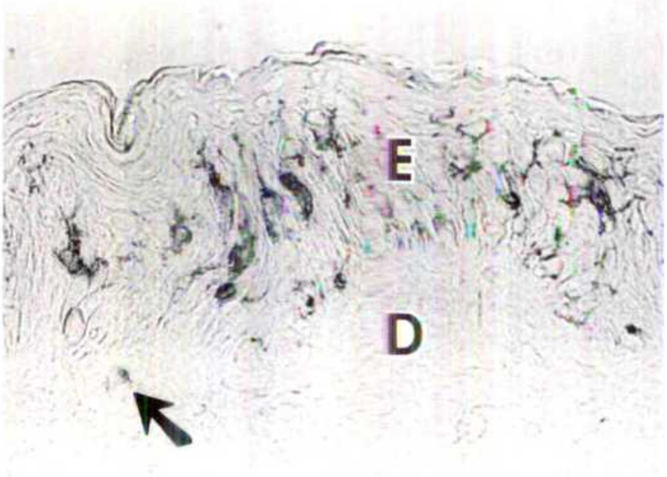

FIGURE 1. Light micrograph of normal epidermis after incubation with anti-T6 antibody. Numerous dendritic cells within the epidermis (E) are T6-positive. A rare, non-dendritic cell in the superficial dermis (arrow, D) is also T6-positive (× 400).

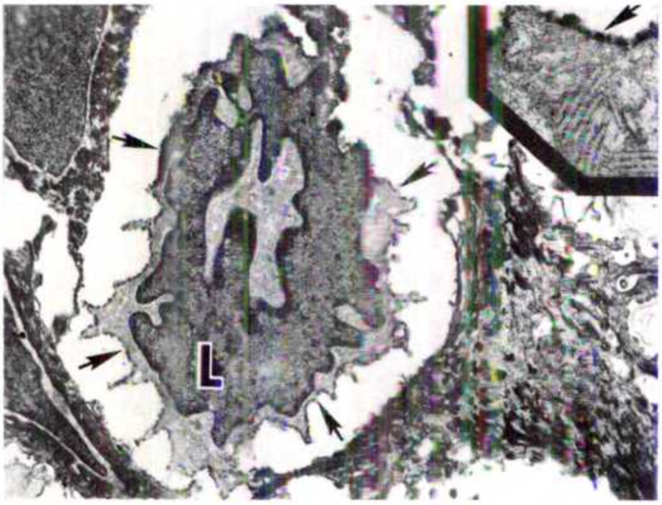

FIGURE 2. Immunoelectron micrograph of Langerhans cells (L) within normal human epidermis incubated with anti-T6 antibody. Note the characteristic Birbeck granules within the cytoplasm (inset) and the deposition of the reaction product of DAB along the cell membrane (arrows) indicating sites of anti-T6 antibody reaction (× 7100, inset × 28,000).

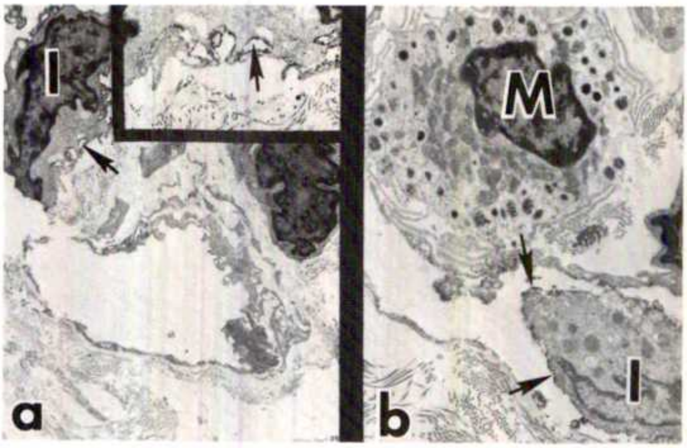

FIGURE 3. Mononuclear cells (I) are present around (3a) and within (3b) dermal lymphatics. The reaction product of DAB is evident along the cell membranes (arrows; 3a, inset), M = mast cell. (3a, × 4100, inset × 8800, 3b, × 5700).

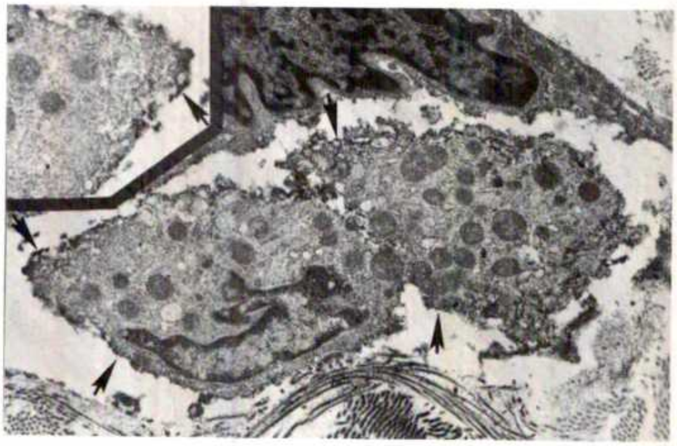

FIGURE 4. A mononuclear cell within a lymphatic vessel contains numerous mitochondria, microfilaments and an infolded nucleus consistent with the structure of an indeterminate cell. Lysosomes or phagolysosomes as seen in dermal histiocytes are not evident. Sites of anti-T6 antibody reaction are evident (arrows). (× 9100; inset × 15,000).

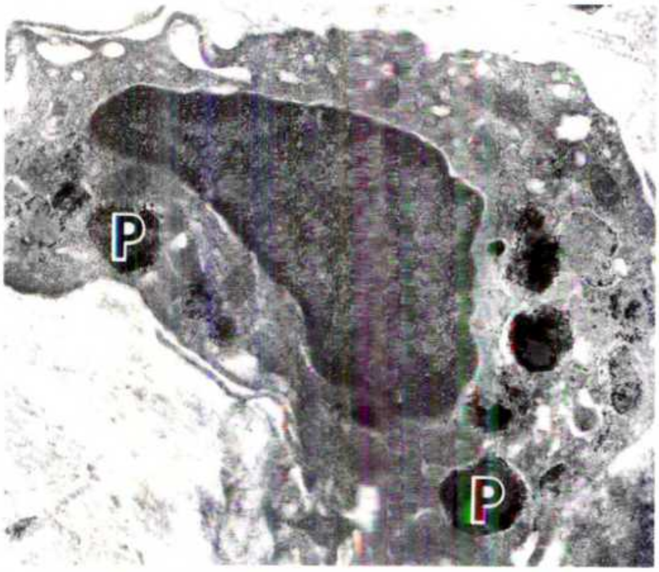

FIGURE 5. A dermal histiocyte contains numerous phagolysosomes (P) and does not react with anti-T6 antibody (× 15,000).

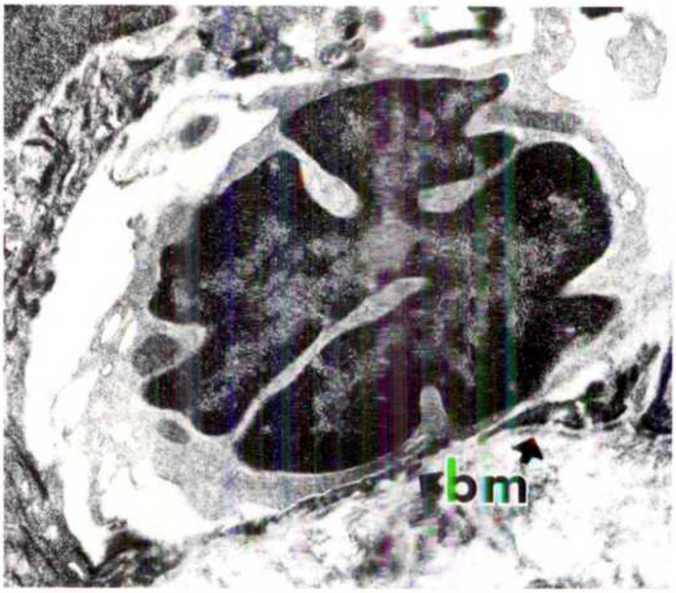

FIGURE 6. A lymphocyte above the epidermal basement membrane (bm) contains a markedly convoluted nucleus and a paucity of organelles. Note absence of DAB reaction product after incubation with anti-T6 antibody (× 15,000).

by anti-I1 antibody. Thus two immunologically distinct mononuclear cell populations are present in normal papillary dermis, namely T6-positive (indeterminate) cells, and T6-negative phagocytic histiocytes.

Lymphocytes within the infiltrates of lichen planus reacted strongly with anti-T3 antibody by light microscopy. Rare dermal and intraepidermal lymphocytes in normal skin (Fig. 6) were characterized ultrastructurally by condensed, peripherally-distributed heterochromatin, frequently infolded or convoluted nuclei and sparse cytoplasm with few organelles. These cells did not react with anti-T6 antibody by immunoelectron or immunolight microscopy. Lymphocytes were not identified in normal tissue incubated with anti-T3 antibody and studied by immunoelectron microscopy.

DISCUSSION

It has been suggested recently on the basis of immunoelectron microscopic observations of epidermal cell suspensions obtained by trypsinization that monoclonal anti-T6 antibody reacts specifically with Langerhans cells and with similar cells with the ultrastructure of indeterminate cells (Chu *et al.*, 1982). Problems inherent in these studies include: (1) the potential destruction (Czop, Fearon & Austen, 1978; Fajumi, 1980; Pinkett & Anderson, 1980) or enhancement (Swoveland & Johnson, 1979) of cell surface antigens due to pre-treatment with trypsin; (2) disproportionate representation of certain cell types due to varying tendencies of different cells to form single cell suspensions; (3) uncertainty concerning the original anatomical relationships of these cells within the epidermis. Furthermore, questions persist regarding the immunological characterization of dermal mononuclear cells in human skin.

Breathnach (1980) has stated that a population of relatively undifferentiated cells in the dermis and Langerhans cells may be related. Because Langerhans cells in the basal layers of the epidermis frequently show a paucity of Birbeck granules (Wolff *et al.*, 1971; Breathnach, 1977), it is reasonable to assume that these cells may represent forms intermediate in the development of structurally-mature Langerhans cells (Rowden, 1980). *In situ* examination of mononuclear cells in the dermis is prerequisite to an understanding of the potential relationship of Langerhans cell precursors to their more mature intraepidermal counterparts as well as to other dermal mononuclear cells, such as macrophages and lymphocytes.

In this study, two immunologically distinct populations of histiocyte-like cells are defined in normal human dermis. Although both types have been previously shown to react with anti-Ia antibodies, only those with ultrastructural features of indeterminate cells react with anti-T6 antibody in a manner analogous to Langerhans cells within the epidermis (Murphy *et al.*, 1981a, b). Thus, these data provide immunological support for the hypothesis that the T6-positive dermal mononuclear cells and Langerhans cell are related.

Langerhans cells migrate by way of dermal lymphatic vessels to regional lymph nodes during contact allergic reactions (Silberberg-Sinakin *et al.*, 1976), and it has been reasoned that the supply of intraepidermal antigen-presenting cells may be in part maintained by a local pool of precursors. In the present study, we have found dermal cells devoid of Birbeck granules but with structural and antigenic similarities to epidermal Langerhans cells. In addition, T6-positive cells within the lower third of the epidermis consistently contained fewer Birbeck granules than those in the middle to upper third. This suggests a possible sequence whereby morphologically less developed precursor cells migrate from the dermis into the epidermis where they acquire structural and perhaps functional maturity. In this case, T6 antigen would be present before cells reach the epidermis, making it unlikely that keratinocytes govern T6 antigen acquisition in a manner analogous to the intrathymic development of T-cell antigens by pro-thymocytes. An alternative possibility, however, is that T6-positive mononuclear cells in the dermis have migrated from the epidermis, as may be inferred from their presence in and around dermal lymphatics. In this latter case, Birbeck granules could either be lost as cells leave the epidermis, or two structurally distinct populations of T6-positive antigen-presenting cells may exist in the skin. Detailed kinetic studies are required to define the dynamic events responsible for repopulation of the epidermis when depleted of structurally mature Langerhans cells.

The data presented herein suggest that T6-positive mononuclear cells without demonstrable Birbeck granules are in fact indeterminate cells. Immunologic detection of T6 cell surface antigen provides a method for the differentiation of these cells from similar mononuclear cells by light and electron microscopy. By light microscopy, T6-positive cells within the skin may represent either Langerhans cells or indeterminate cells. Ia-positive cells, however, may represent Langerhans cells (Rowden *et al.*, 1977), indeterminate cells (Rowden *et al.*, 1979), phagocytic histiocytes, B cells or activated T cells (Rowden, 1980). Because of these observations, we recommend caution in intrepreting Ia-positive cells in the skin as Langerhans cells or their derivatives without immunoelectron microscopic confirmation. Similarly, coincident Ia-positivity by light microscopy alone does not constitute proof that T6-positive cells in the skin are Langerhans cells.

Several recent studies have noted increased numbers of T6-positive cells in the dermis of lesions of lichen planus (Bhan *et al.*, 1982), erythema multiforme (T.J.Harrist *et al.*, personal observation, 1982) and lymphomatoid papulosis (Harrist *et al.*, 1981). By immunoelectron microscopy, the presence of T6 antigen on indeterminate cells allows differentiation from dermal histiocytes and large activated T cells. These specific applications may be limited to benign inflammatory conditions in the skin, since neoplastic T-cell lines may demonstrate T6 surface antigen (Terhorst *et al.*, 1981).

In summary, the reactivity of dermal mononuclear cells with monoclonal anti-T6 antibody provides further immunological support for the concept that Langerhans cells and indeterminate cells are related. Evidence is accruing that indeterminate cells within the epidermis or dermis may represent a precursor pool for antigen-presenting cells within the epidermis. Detection of T6 surface antigen provides a probe for the differentiation of these cells form morphologically similar mononuclear cells in the skin.

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A new way to evaluate the germinative compartment in human epidermis, using [^{3}H]thymidine incorporation and immunoperoxidase staining of 67 K polypeptide

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SUMMARY

We have used tritiated thymidine (^{3}HT) labelling and immunoperoxidase staining of 67 K polypeptide in human epidermis to study normal skin before and after irritation induced by application of croton oil (20% and 50% in olive oil). By this double labelling method, it was possible to identify, on the same skin section, DNA-synthesizing nuclei and epidermal cells which contained 67 K polypeptide.

Our results clearly indicate that the germinative compartment of normal epidermis is a mixed population comprising basal and adjacent suprabasal cells; as a rule, absence of expression of 67 K polypeptide, which characterizes all basal cells, could not be regarded as a good marker to distinguish the germinative from the differentiating compartments.

In mild primary irritation, the ratio of ^{3}HT-labelled undifferentiated cells to keratinizing ones was similar to that observed in normal epidermis. In severe irritation, this homeostatic process was altered, as ^{3}HT-labelled cells were mainly 67 K negative.

Several methods have been developed to study cell behaviour in the epidermis by identifying proliferating (Laurence, Spargo & Thornley, 1981) and differentiating (Bystryn, 1977; Potten, 1981) epidermal cells. Morphological evaluation of the epidermal proliferative process has usually been approached by means of descriptive histology and/or by quantification of cells in mitosis (M phase) or in DNA synthesis phase (S phase). One of the main drawbacks of a very sophisticated technique, e.g. DNA flow cytometry (Bauer & De Grood, 1975, 1976; Frentz, Müller & Christensen, 1980), is that information about localization of proliferating cells is lost.

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This point is of importance as, after cell division, epidermal cells move to the epidermal surface and finally lose their proliferating capacity. The migration process is also characterized by a gradual modification of the cellular keratin content. Indeed, the molecular weight of the synthesized keratin polypeptides increases, and, in our hands, high molecular weight keratin polypeptides (mol. wt 67,000 D or 67 kD or 67 K) were never present in basal cells (Viac *et al.*, 1980b). Since expression of 67 K polypeptide is diminished in hyperproliferative skin tumours (Viac *et al.*, 1980b) we have tried to use 67 K antisera to distinguish the differentiating from the germinative compartment.

In this paper, we describe how to combine [^{3}H]thymidine (^{3}HT) labelling and immunoperoxidase staining of 67 K polypeptide in human epidermis and we report the first results obtained in normal skin and the changes occurring after irritation induced by application of croton oil. By this double labelling method, it is possible to identify, on the same skin sections, DNA-synthesizing cell nuclei of the germinative layers and epidermal cells engaged in the differentiation process and expressing 67 K polypeptides.

METHODS

Twelve skin specimens were obtained from four patients (three females, one male; mean age: 81 years) between 9 and 10 a.m. in order to avoid diurnal fluctuations of DNA synthesis (Schell *et al.*, 1981). They were taken after local anaesthesia (2% lignocaine without adrenaline) from the upper part of the back, 45 min after intradermal injection of 0·1 ml of ^{3}HT (10 μCi, specific activity 10 Ci/mmole, Centre d'Energie Nucléaire, Mol, Belgium) according to the technique of Lachapelle (1970).

Two levels of irritation were obtained by pretreatment of the skin with two concentrations of croton oil (20% and 50% in olive oil) left under occlusion (Leukotest®, Beiersdorf) for 48 h. Tests were applied at the upper part of the back and symmetrical untreated sites served as controls.

67 K antiserum

67 K antiserum was obtained by immunizing Hartley guinea-pigs (400 g) with 67 K polypeptides. These polypeptides were extracted from normal human corneocytes as described in detail by Viac *et al.* (1980a). Briefly, horny cells were obtained by scraping foot callus of healthy subjects. After keratin extraction (Baden *et al.*, 1978) and separation of fibrous proteins by 12% polyacrylamide slab gel electrophoresis, the bands containing 67 K polypeptides were used for immunization. Specificity of this 67 K antiserum was controlled by light and electronmicroscopic studies (Viac *et al.*, 1980a).

Immunoperoxidase staining

Throughout this study we used a 1 : 20 dilution of 67 K antiserum. Serial skin sections were left in contact for 30 min with this antiserum. Subsequently they were washed for 30 min in PBS and then treated for 30 min with peroxidase-labelled rabbit anti-guinea-pig Ig (1 : 50 dilution; Nordic). Following two successive washes (30 min in PBS and 10 min in TRIS-HCl buffer, pH 7·6), the presence of peroxidase was revealed by a 15 min contact with Graham–Karnovsky medium (Graham & Karnovsky, 1966). Slides were then rinsed in TRIS-HCl (pH 7·6), PBS, and finally processed for light microscopy radioautography.

Fixation

In a series of preliminary experiments, we established that labelling of 67 K polypeptides in human epidermis by immunoperoxidase technique remained unaffected after fixation of skin specimens in Bouin's fluid. Hence, after fixation and dehydration, paraffin-embedded specimens were serially sectioned, mounted on glass slides and processed for immuno-labelling and peroxidase staining.

Historadioautography

The peroxidase staining was not altered by light microscopy radioautography. This became evident after comparative examination of serial sections of which one was treated and the other not treated for radioautography. (Figs 1a, 1b).

Morphometric analysis

For each specimen, we determined the following:

1) Length of the biopsy at the epidermal surface (stratum corneum, SC).
2) Length of dermoepidermal junction (DEJ).
3) Number of basal cells (not stained by immunoperoxidase technique).
4) Number of suprabasal cells (immunoperoxidase-stained cells; granular layers and overlying corneocytes not included).
5) Number of ^{3}HT-labelled basal cells (immunoperoxidase negative and ^{3}HT positive).
6) Number of ^{3}HT-labelled suprabasal cells (immunoperoxidase positive and ^{3}HT positive).

Mean length of specimens at the epidermal surface was 2·87 mm (range: 2–3·6 mm).
Mean number of epidermal cells counted was 1633 (range: 1225–2166).

From these data, we calculated the following:

1) Number of ^{3}HT-labelled cells per mm length of DEJ.
2) Number of ^{3}HT-labelled basal cells per mm length at the epidermal surface (SC).
3) Number of ^{3}HT-labelled suprabasal cells per length of DEJ.
4) Number of ^{3}HT-labelled suprabasal cells per length at the epidermal surface (SC).
5) Basal labelling index: $(a5/a3) \times 100$.

Statistical analysis

For comparison between means we used Student's t-test; P values larger than 0·05 (bilateral test) were considered not significant; otherwise, levels of significance will appear in the text.

RESULTS

Immunoperoxidase staining of cells expressing 67 K polypeptide in normal epidermis allowed a clearcut distinction between the basal compartment where no staining was present and the suprabasal compartment where the cells showed dark stain within cytoplasm (Figs 2 & 3). ^{3}HT-labelled cells were mainly present in the basal compartment and in the lowest rows of suprabasal cells. The mean basal and suprabasal ^{3}HT-labelling indices were 2·51% and 1·71% respectively. The mean ratio between ^{3}HT-labelling indices of basal and epibasal cells calculated from individual values was 1·95 (Table 1); this means that two-thirds of ^{3}HT-labelled cells did not express 67 K polypeptide and one third were simultaneously labelled by nuclear ^{3}HT and cytoplasmic immunoperoxidase stain. Results of cell counts are detailed in Table 1.

At first glance, in sites where strong irritation had been induced, severe alterations of the

TABLE 1. Results of epidermal cell counts in normal skin before and after irritation induced by croton oil, showing mean number (± standard deviation) of total cell (^{3}HT-labelled and unlabelled) and of ^{3}HT-labelled cells,* expressed as number/unit length of dermo-epidermal junction in epidermis (E). Epidermal cells are further divided depending on the absence of 67 K polypeptide (basal cells, B) or its presence (suprabasal cells, SB). Basal labelling index (BLI) and suprabasal labelling index (SBLI), relative proportion of ^{3}HT-labelling indices of basal cells to epibasal cells (BLI/SBLI; calculated from individual values) and relative length of dermo-epidermal junction to stratum corneum (DEJ/SC) are shown in the four last rows

	Normal skin	20% Croton oil	50% Croton oil
E	428·81 ± 73·08	418·35 ± 35·19	392·15 ± 86·23
B	169·09 ± 25·48	158·50 ± 11·38	173·22 ± 14·13
SB	262·13 ± 45·24	260·35 ± 28·43	219·57 ± 74·78
E*	8·67 ± 3·38	40·21 ± 19·33	59·25 ± 20·98
B*	4·03 ± 1·6	18·99 ± 13·59	41·77 ± 13·96
SB*	4·64 ± 2·7	21·22 ± 5·74	17·46 ± 16·36
BLI	2·51 ± 1·20	11·93 ± 8·05	23·94 ± 6·93
SBLI	1·71 ± 0·82	8·11 ± 1·62	6·92 ± 4·69
BLI/SBLI	1·95	1·37	4·58
DEJ/SC	1·29	1·39	1·59

superficial epidermis, generally around adnexae (Fig. 1), obscured cell limits; such areas were not considered for future cell counting. Our first impression was that there could be an overall increase of the basal compartment (Fig. 3b). However, this was not supported by objective findings; indeed total numbers of epidermal, basal, or suprabasal cells showed no significant modification as compared to control skin nor did lengths of dermoepidermal junctions (Table 1).

FIGURE 1. Immunoperoxidase labelling of 67 K polypeptide (a and b) and historadioautography (b) in croton oil-induced irritation.

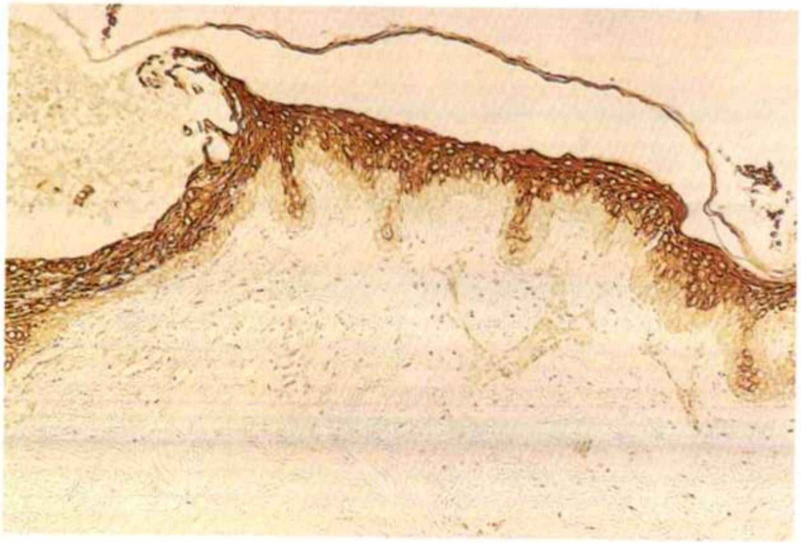
Adjacent sections (a) and (b) were processed for immunoperoxidase staining of 67 K polypeptide (these cells stained brown). Immunoperoxidase staining seems to be unaffected by further treatment of section (b) by historadioautography. Hence ^{3}HT-labelled cells (black dots) and cells engaged in the differentiation process (67 K positive cells) can be observed on the same section. Periporal pustule formation (on the left) is usually present in severe croton oil-induced irritation. These areas where cellular limits were obscured were not considered for cell counting.

FIGURE 2. Immunoperoxidase staining of 67 K polypeptide and ^{3}HT labelling in normal human skin.

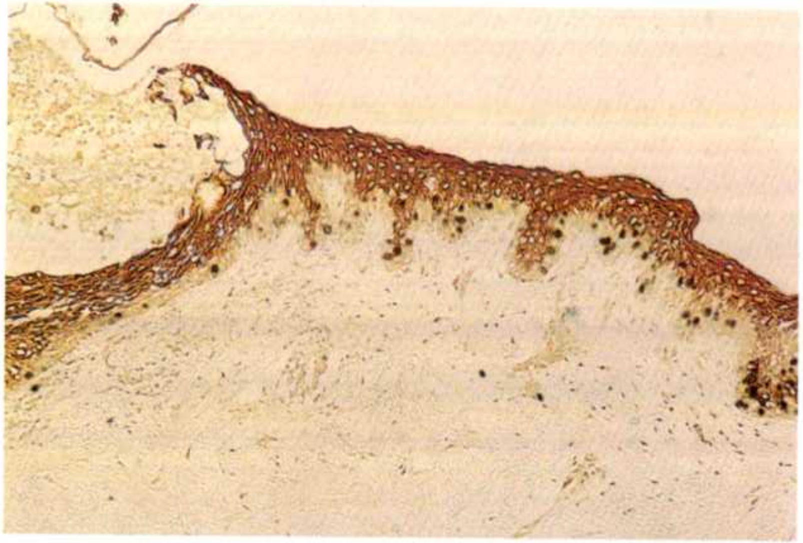
Immunoperoxidase staining of 67 K polypeptide is exclusively present in suprabasal epidermal cells, leaving basal cells unstained in normal human epidermis. ^{3}HT-labelled cells were observed in basal (67 K negative) and in suprabasal (67 K positive) cells.

FIGURE 3. Patterns of ^{3}HT labelling and immunoperoxidase staining of 67 K polypeptide in croton oil (20% (a) and 50% (b)) induced irritation (same patient and identical magnification as Fig. 2).

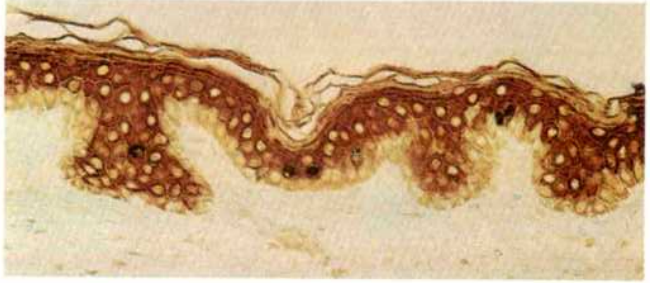
Mild (a) and severe (b) irritation induced a clearcut increase in numbers of ^{3}HT-labelled cells. In mild irritation (a) the ratio of ^{3}HT-labelled basal/^{3}HT-labelled suprabasal cells was as in control skin. In severe irritation (b) ^{3}HT-labelled cells were merely 67 K negative cells.



1a



1b



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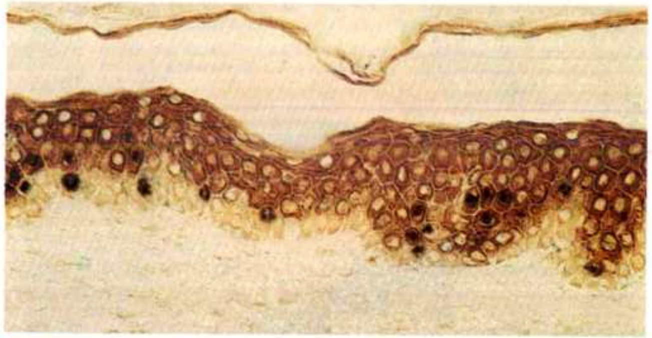
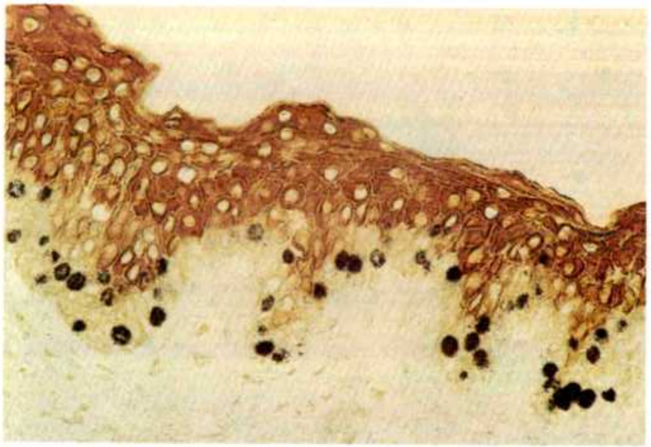


3a



3b

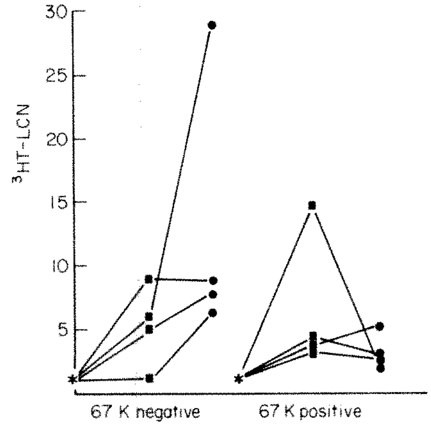


FIGURE 4. Individual values of ³HT-labelled cells per mm length of dermo-epidermal junction.

For presentation purposes, ³HT-labelled cell numbers (³HT-LCN) after irritation by 20% (■) or 50% (●) croton oil are shown relative to control skin (1 = arbitrarily chosen normal value (∗)). ³HT-labelled cells are separated into 67 K negative and 67 K positive depending on the absence or presence of immunoperoxidase labelling of 67 K polypeptide in cytoplasm.

Similarly, numbers of cells in each compartment did not vary significantly between treated and untreated sites when values were related to length of DEJ or SC. The most clearcut change was a tremendous increase in ³HT-labelled cells per unit length of dermo-epidermal junction. These cells occurred in basal and suprabasal ($P < 0{\cdot}005$) populations in mild irritation (20% croton oil, Fig. 3a), whereas severe irritation (50% croton oil, Fig. 3b) produced an increment of only ³HT-labelled basal cells ($P < 0{\cdot}005$) as compared with control skin. The ratio of ³HT-labelling indices of basal to suprabasal cells populations (Table 1) particularly illustrates the fact that in severe irritation more than 80% of ³HT-labelled cells are actually undifferentiated (67 K negative basal cells), compared with around 60% after mild irritation ($P < 0{\cdot}05$) or in untreated skin. Individual variations are shown in Fig. 4.

DISCUSSION

The keratinization process occurs in epidermal cells migrating from the proliferative (or germinative) compartment of the epidermis into the overlying epidermal layers. This migration process is associated with a series of morphological and biochemical changes. Several authors have suggested that detection of epidermal cytoplasmic antigens by using naturally occurring antibodies (Bystryn, Abel & Weidman, 1973; Burnham, 1974; Van Joost, 1974; Saurat *et al.*, 1978; Saurat, Didierjean & Habibi, 1981), or experimentally produced anti-polypeptide antisera (Viac *et al.*, 1980a, b; Loening *et al.*, 1982), could be valuable markers of epidermal differentiation. Furthermore, at the same time they could be helpful in defining what has been called 'the germinative compartment of the epidermis'. To test this hypothesis, we combined [³H]thymidine flash-labelling as a marker of DNA synthesis (Laurence *et al.*, 1981) and immunoperoxidase stain of 67 K polypeptide as a marker of cells engaged in the differentiation process (Viac *et al.*, 1980a, b). Furthermore, to get significant information from this experimental *in vivo* work, we compared symmetrical sites of the upper part of the back where biopsies from normal skin and from the sites of primary irritant dermatitis were taken. Indeed, croton oil-induced injury is a well-known model of primary irritancy and is accompanied by increased epidermal DNA synthesis in both animals (Hennings, Bowden & Boutwell, 1969) and humans (Van Neste & Lachapelle, 1981).

The finding of a significant percentage of ^{3}HT-labelled cell nuclei in the supra-basal layers is not surprising as it had already been noted by Penneys *et al.* (1970). Three-dimensional localization of DNA-synthesizing cells by superimpression of drawings made from adjacent sections (Penneys *et al.*, 1970) gives approximately the same results as the presently described method. Indeed, in normal human epidermis, the ratio of ^{3}HT-labelled basal to supra-basal cells calculated on the basis of their published data and from our observations are 2·12 and 1·95 respectively.

As modifications of polypeptide composition may occur under pathological conditions (Baden *et al.*, 1978; Baden, Kubilus & Argyris, 1980; Staquet, Viac & Thivolet, 1981; Viac *et al.*, 1980a,b) it seems reasonable to restrict the use of immunoperoxidase labelling of 67 K polypeptide as a marker of cells that have lost contact with basement membrane zone only for specimens of normal skin.

In experimentally induced reactive processes, the dramatic increase of ^{3}HT-labelled cell nuclei was in sharp contrast with the apparent absence of significant modification of the other variates (i.e. total numbers of epidermal, basal, suprabasal cells, and relative length of dermo-epidermal junction). It is noteworthy that in mild irritation, DNA synthesis took place in basal and suprabasal cells in the same proportion as in control skin, whereas in severe irritation the bulk of DNA-synthesizing cells occurred in the basal cell population. As stated earlier in this discussion, we consider absence of expression of 67 K polypeptide as a marker of basal cells from the physiological and not from the stereological point of view. Nevertheless, by observation of adjacent sections, we gained the impression that apparently suprabasal localization of 67 K negative cells (Fig. 3b) could partly result from stretching of the epidermal rete pegs by the inflammatory process, but the exact proportion of truly basal (i.e. in contact with basement membrane zone) and truly suprabasal (67 K negative cells which lost contact with BMZ) remains to be evaluated from immuno-electronmicroscopic studies.

From the present work, we conclude that:

(1) In normal skin (a) expression of 67 K polypeptide in epidermal cell cytoplasm is not valuable as a positive marker of cells irreversibly engaged in the differentiation process and as a rule it would not be usable as a negative marker of the germinative compartment. (b) DNA-synthesizing nuclei are distributed closely along the limit between basal and suprabasal compartment.

(2) In mild primary irritant dermatitis, there is a recruitment of suprabasal cells joining the cycling pool but relative proportions of ^{3}HT-labelled cells with or without expression of 67 K polypeptide in the cytoplasm remain as in control skin.

(3) In more severe irritation, the steady state is disrupted and one observes a shift of ^{3}HT labelling from 67 K positive toward 67 K negative cells.

Combination of other immunological tracers with ^{3}HT incorporation could be useful in the study of trigger mechanisms of epidermal hyperplasia and to identify cycling and non-cycling cells (Wright, 1977) present in the epidermal proliferative unit (Potten, 1981).

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Specific incorporation of penicillamine into the epidermis of mice: an autoradiographic study

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SUMMARY

[^{3}H]-D-penicillamine was injected intra-peritoneally in mice. The highest concentration of the drug was found in the skin, particularly in the epidermis, and a well-defined kinetic sequence of appearance in the epidermis was shown. This finding is consistent with the hypothesis that penicillamine, by virtue of its chemical similarity to cysteine, can replace the latter during keratogenesis by a competition phenomenon, which could provoke acantholytic splitting both biochemically and immunologically.

Penicillamine is known to provoke acantholytic splits of human epidermis both *in vivo*, by systemic administration (penicillamine-induced pemphigus) (Marsden *et al.*, 1976), and *in vitro*, by addition to normal human serum during culture of normal human skin explants (Ruocco *et al.*, 1982). As acantholytic activity of penicillamine does not seem to be antibody dependent, and considering the close structural similarity between the drug and cysteine (Fig. 1), the hypothesis has been advanced that penicillamine may substitute for cysteine during keratogenesis by a competition mechanism, impairing epidermal intercellular cohesion and producing acantholytic splitting (Ruocco *et al.*, 1979).

The aim of the present work was to investigate whether systemic administration of penicillamine was followed by high drug concentration in the skin, and to localize by autoradiography the distribution of the drug within the skin.

MATERIALS AND METHODS

[^{3}H]-D-penicillamine (ICN Pharmaceuticals, Irvine, California, U.S.A.) of 0·22 mCi/mg specific activity, was injected intraperitoneally into five groups of C3H mice (two animals/

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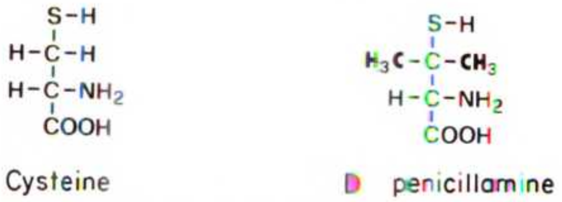

FIGURE 1. Structural similarities between cysteine and D penicillamine.

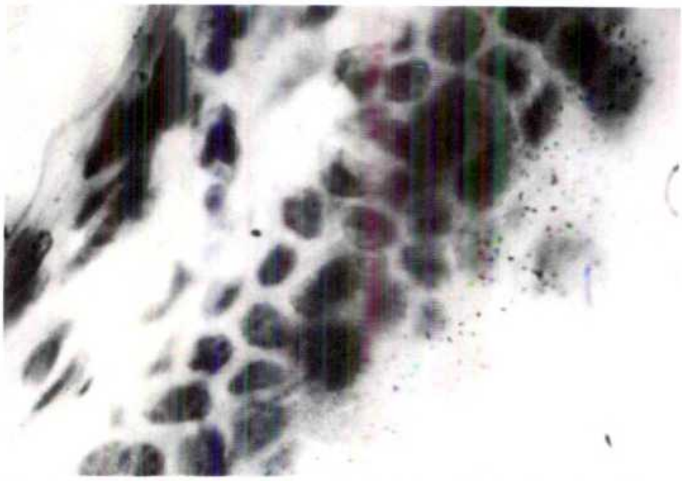

FIGURE 2. Labelling confined to dermis 4 h after [^{3}H]penicillamine injection (H & E, × 1130).

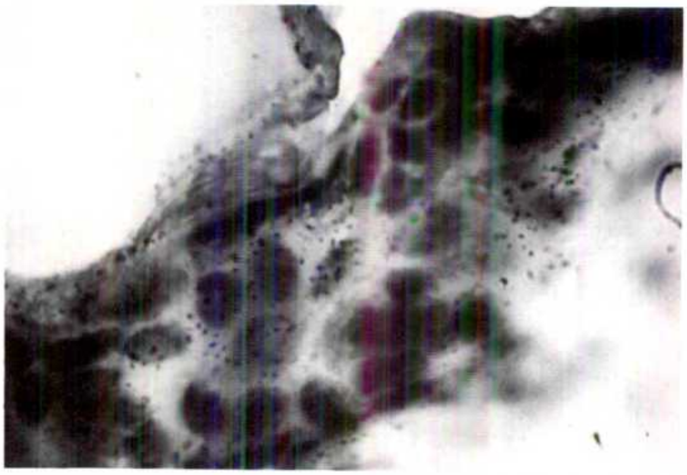

FIGURE 3. Labelling in the epidermis 8 h after [^{3}H]penicillamine injection (H & E, × 1130).

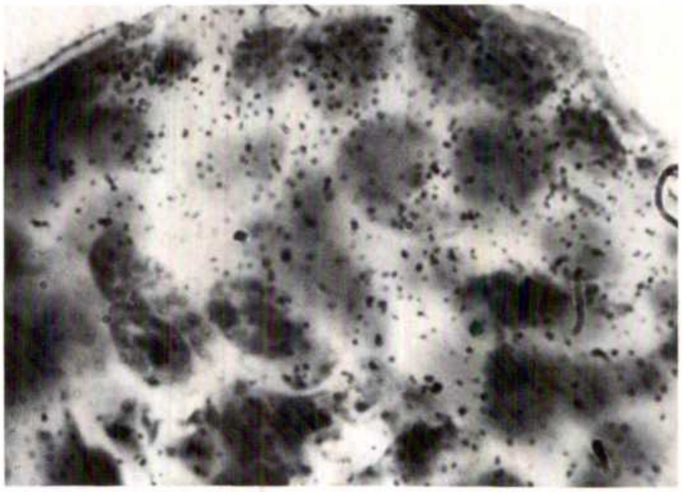

FIGURE 4. High magnification of epidermal labelling (H & E, × 2000).

group) at a concentration of 220 μg of the drug and 50 μCi/mouse. Mice were sacrificed at 2, 4, 8, 24 and 48 h after injection by cervical dislocation under ether anaesthesia. The skin of the ear, abdomen and back, together with several internal organs, were collected and fixed for 24 h in neutral buffered formalin. After embedding and sectioning, 4-μ sections were submitted to autoradiography by dipping in 1:1 dilution of Ilford K5 nuclear track emulsion (Ilford, Essex, England). Slides were developed in Kodak D 19 developer after 3 weeks of exposure and stained with haematoxylin and eosin.

RESULTS

Microscopic observation of the slides showed very low labelling of certain internal organs, such as the kidney and submaxillary gland, while high labelling was observed in the skin. In the epidermis there was much more than in the dermis and subcutis.

Labelling was negligible in the skin at 2 h after injection, weak and confined to the dermis (Fig. 2) and subcutis at 4 h, strong in the epidermis (Figs 3 & 4) and weak in the dermis and subcutis at 8 h. Within the epidermis, the upper layers showed a higher concentration of silver grains than in the lower layers. In mice sacrificed 24 and 48 h after the injection the labelling of the whole cutis was weaker than in the 8 h specimens.

DISCUSSION

The high concentration of labelled penicillamine found in the skin provides further evidence of the drug's tropism toward the whole cutis (epidermis, dermis and subcutis) already shown by liquid scintillation counting (Planas-Bohne, 1972). In this report evidence is given by autoradiography that within the skin the highest concentration of penicillamine is attained in the epidermis and a well-defined kinetic sequence of its appearance is seen.

The peculiar epidermal tropism of penicillamine seems to support the hypothesis that the drug may provoke a pemphigus-like syndrome by interference with the epidermal biochemistry. The formation of anomalous prekeratin proteins, incorporating penicillamine instead of cysteine molecules, would lead to alterations of the biochemical and antigenic structure of epidermal cell membranes. The acantholytic effect of the drug could be due to impairment of intercellular cohesion (*biochemical acantholysis*) as well as to mediation of antibodies secondarily elicited by antigenically anomalous proteins (*immunological acantholysis*). That only some of the many patients subjected to long-term penicillamine administration develop a pemphigus-like syndrome or a true pemphigus might be due to lability of membrane enzymes and/or immunological imbalance in such subjects.

Antibody-unrelated acantholysis provoked by penicillamine, which has been demonstrated *in vitro* (Ruocco *et al.*, 1982) as well as *in vivo* (Troy *et al.*, 1981), may occur by the above mechanism. This could explain the repeated observation that intercellular antibodies, unlike the situation in idiopathic pemphigus, do not play a role of primary importance in penicillamine-induced pemphigus.

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Abnormal lymphocyte function following long-term PUVA therapy for psoriasis

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SUMMARY

The surface markers and function of peripheral blood lymphocytes were examined in patients on long-term therapy with methoxsalen and UV-A radiation (PUVA). Ten patients with psoriasis were selected because they had received a high exposure to PUVA therapy, i.e., more than 200 treatments over 2–6 years with cumulative exposure doses of 1700–6000 J/cm^2 UV-A radiation. Results were compared to those obtained with lymphocytes from untreated patients and UV-B treated patients with psoriasis. The PUVA-treated patients had low levels of E rosette-forming cells in the peripheral blood and markedly impaired lymphocyte responses following stimulation with optimal and suboptimal doses of mitogens. The sensitivity of lymphocytes to *in vitro* treatment with PUVA was similar in the three groups of patients. The results of this study indicate that long-term PUVA therapy alters the function and cell-surface markers or distribution of lymphocytes.

The effect of PUVA therapy on immune function has attracted much interest and some short-term alterations of lymphocyte function and other immune functions have been reported; this subject has been reviewed recently (Morison, 1981). However, only one study of the long-term effects of PUVA therapy on lymphocyte function has been reported (Harper *et al.*, 1979). In that study patients on maintenance therapy with controlled psoriasis were examined after receiving PUVA therapy for 6–33 months. The responses of peripheral blood lymphocytes to mitogens and the proportion of E rosette-forming lymphocytes in the peripheral blood of these patients were normal. The present study was designed to examine lymphocyte function in patients who had received larger cumulative exposure doses of PUVA therapy. Patients who had received the most PUVA treatment were selected from several hundred patients being followed in our PUVA therapy unit. Lymphocyte function in these patients was compared with that in patients with psoriasis who had not received PUVA therapy or intensive

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UV-B phototherapy. We also studied patients who had received the highest doses of UV-B phototherapy.

METHODS

Patients

Three groups were examined.

Untreated. This group consisted of eleven patients (mean age 32 years, range 13–43 years) with active, spreading psoriasis (10–70% body surface) who had not received PUVA therapy or UV-B phototherapy. Some of the patients had received topical corticosteroid therapy, but not during the 2 weeks prior to testing.

PUVA. Ten patients with psoriasis (mean age 48 years; range 28–72 years) who had received more than 200 exposures to PUVA therapy (mean 272, range 205–340) and a mean total cumulative exposure to UV-A radiation of 4150 J/cm^2 (range 1700–7600 J/cm^2) over 2–6 years were in this group. These patients were selected on the basis that they all had marked changes of premature ageing of their skin. The disease was controlled at the time of testing and all patients were on maintenance therapy.

UV-B. In this group there were ten patients with psoriasis (mean age 40 years, range 21–65 years) who had received a mean of 125 (range 62–210) exposures to intensive UV-B phototherapy over 1–2 years. These patients had received the highest exposure doses of UV-B in our population of patients.

All patients were on maintenance therapy at the time of examination and had well-controlled disease.

In vitro studies

All tests were performed at least 4–7 days after the preceding exposure to PUVA or UV-B phototherapy. Twenty to 40 ml of venous blood were collected in a heparinized syringe. After white blood cell and differential counts were performed, the mononuclear cells were separated on a Ficoll–Hypaque gradient and tested as follows.

Enumeration of T cells. The E rosette test was used as a marker for T lymphocytes. Mononuclear cells were incubated with latex particles (Difco, Detroit, MI) for 45 min, washed and a sample was mixed with sheep erythrocytes (Becton Dickinson, Cockeysville, MD) plus 10% fetal calf serum (FCS) (Gibco, Grand Island, NY) to yield an erythrocyte to lymphocyte ratio of 80:1. The cell suspension was incubated at 37°C with 5% CO$_2$ for 15 min, centrifuged at 4°C for 5 min at 200 g and then incubated at 4°C overnight. Cells were resuspended by gentle tapping of the tube and the rosette-forming lymphocytes (lymphocytes with three or more adherent erythrocytes) were counted in a haemocytometer. Cell viability was evaluated by trypan blue dye exclusion; 100 viable cells were counted, and the number of E rosettes was expressed as a percentage of viable latex-free mononuclear cells. The absolute number of E rosette-positive cells was calculated from the lymphocyte count. The study was performed in triplicate and the results were averaged.

Mitogen stimulation. Cultures of mononuclear cells were established in microtitre plates and incubated with various amounts of phytohaemagglutinin (PHA) (purified HA 17; Burroughs Wellcome, Beckenham, England), concanavalin-A (con-A) (Miles Yeda, Elkhart, IN), or pokeweed mitogen (PWM) (Gibco). Each culture contained 2×10^5 cells in RPMI (Gibco) with 10% FCS and antibiotics (penicillin 200 iu/ml and streptomycin 200 μg/ml). Six concentrations of each mitogen were used (PHA: 10, 1·0, 0·5, 0·25, 0·1 and 0·01 μg/culture; con-A: 27, 9, 3, 1·0, 0·33, 0·11 μg/culture; PWM: 20, 10, 5, 1·0, 0·10, 0·01 μg/culture) in order to yield a range of stimulating doses above and below the optimal stimulating doses (PHA: 0·25 μg; con-A: 3·0 μg; PWM: 1·0 μg) determined in preliminary experiments. All cultures were established in triplicate. After 66 hr incubation at 37°C with 5% CO_2 0·5 μCi of tritiated thymidine (sp. act. 6·7 Ci/mM; New England Nuclear, Boston, MA) was added and the cultures were incubated for a further 6 hr. The cell cultures were harvested using an automated harvester (MASH, Microbiological Associates, Walkersville, MD) and the radioactivity of each sample was measured in a liquid scintillation spectrophotometer (Beckman, Model LS 150, Wakefield, MA). The incorporation of tritiated thymidine into DNA was expressed as c.p.m./culture.

In vitro exposure to PUVA. Mononuclear cells were suspended in Hank's balanced salt solution, without phenol red, at a concentration of 2×10^6/ml in 3 ml samples. Methoxsalen was added to yield a final concentration of 1 μg/ml; the suspension was then incubated for 15 min at 37°C with 5% CO_2. Thereafter, the cell suspension was irradiated from above in 3·5 cm open petri dishes placed on a nonreflecting surface. Cells were subjected to graded doses of UV-A radiation ($0{\cdot}0$–2×10^3 J/m^2) to determine a dose–response curve. Three controls were included in each experiment: cells exposed to psoralen but not UV-A radiation, cells exposed to the highest UV-A dose without psoralen, and cells kept under ambient lighting and not exposed to psoralen or UV-A radiation. Following irradiation, the cells were washed, resuspended in RPMI and 10% FCS and antibiotics and stimulated with 0·25 μg of PHA per culture. The remaining culture conditions were as described above. The mean of the results (c.p.m./culture) of triplicate cultures was expressed as a percentage of the control cultures which were not exposed to radiation or psoralen.

The source of UV-A radiation was provided by passing the radiatively-collected radiant output of a 1·0 kW Xenon arc lamp through 10 cm of circulating solution (40 g $CuSO_4$ and 40 g $CoSO_4$ per liter of distilled water). The spectral irradiance of the system was essentially limited to the 320–400 nm waveband with a peak at 365 nm. The details of this radiation procedure have been described (Morison *et al.*, 1982).

Differences in the results obtained among the three groups of patients were analysed using the two-tailed Student's t test; a P value $< 0{\cdot}05$ was regarded as significant.

RESULTS

The proportion and absolute number of circulating E rosette-forming lymphocytes were significantly reduced in the PUVA-treated patients as compared with untreated and UV-B-treated patients (Table 1). The absolute number of lymphocytes was also lower in PUVA-treated patients but this difference was not significant. The proportion of lymphocytes to other leukocytes was similar in the three groups of patients, so that the total white blood cell count was lower in PUVA-treated patients as compared with the other groups of patients.

The responses of lymphocytes following stimulation with graded doses of PHA and PWM were significantly impaired in the PUVA-treated patients as compared with the responses in untreated and UV-B-treated patients (Table 2). The responses of lymphocytes following stimulation with con-A were also lower in the PUVA-treated patients but this reduction was not significant.

Treatment of lymphocytes with methoxsalen and UV-A radiation *in vitro* did not reveal any significant differences in the subsequent response to an optimal dose of PHA among the three groups of patients. The UV-A radiation doses required to reduce PHA-stimulated incorporation of [^{3}H]thymidine by 50% in the presence of 1 μg/ml of methoxsalen were: untreated patients, 904 J/m^2; PUVA-treated patients, 1130 J/m^2; and UV-B treated patients, 1038 J/m^2.

TABLE 1 Distribution of lymphocytes and T cells in peripheral blood

Patients	% Lymphocytes (±s.d.)	Absolute number of lymphocytes/mm^3 (±s.d.)	% T cells (±s.d.)	Absolute T cells/mm^3 (±s.d.)
Untreated	29 (±8)	2233 (±[illegible]66)	70 (±8)	1532 (432)
PUVA	30 (±9)	1791 (±[illegible]13)	62 (±9)	1148 (±558)*
UV-B	35 (±8)	2303 (±[illegible]85)	70 (±8)	1615 (±768)

* The difference between untreated and PUVA-treated patients was significant ($P < 0{\cdot}05$).

TABLE 2. Mean responses of peripheral blood lymphocytes to mitogen stimulation

Mitogen (μg/culture)		Patient group		
		Untreated	PUVA	UV-B
PHA	10	3·111*	2·[illegible]16	3·760
	1·0	4·568	3·[illegible]61†	4·574
	0·5	4·587	4·[illegible]63†	4·609
	0·25	4·564 (±0·205)	4·[illegible]01 (±0·372)†	4·594 (±0·260)
	0·1	4·460	4·[illegible]11†	4·506
	0·01	2·168	2·[illegible]43	2·973
Con-A	27	2·913	2·[illegible]92	2·582
	9	4·036	3·[illegible]66	4·210
	3	4·486 (±0·322)	4·[illegible]51 (±0·449)	4·452 (±0·432)
	1·0	4·040	3·[illegible]44	4·035
	0·33	3·000	2·[illegible]25	3·095
	0·11	1·921	1·[illegible]75	2·357
PWM	20	3·831	3·[illegible]13†	3·667
	10	4·071	3·497†	4·116
	5	4·179	3·[illegible]15†	4·236
	1·0	4·174 (±0·239)	3·[illegible]90 (0·385)†	4·233 (0·425)
	0·10	3·955	3·[illegible]42†	3·834
	0·01	3·311	1·[illegible]50†	2·260†

* Geometric mean of C.P.M. per stimulated culture minus C.P.M. per unstimulated culture.

† The difference between untreated and PUVA-treated patients was significant ($P < 0{\cdot}05$).

s.d. (in parentheses) is shown for optimal mitogen concentrations only.

DISCUSSION

This study has demonstrated that patients with psoriasis treated with large cumulative doses of PUVA therapy resulting in evidence of cutaneous damage have a decrease in circulating T lymphocytes and a decreased response of their lymphocytes to mitogens. In a parallel study of the peripheral blood T-cell subset distribution in the same patients, it was found that the proportion of T4-positive (helper/inducer) lymphocytes was reduced in the PUVA-treated patients (Moscicki *et al.*, 1982). The finding of a reduction of circulating helper/inducer cells correlates with the functional impairment of mitogen responses observed in the present study.

The alteration in the distribution and function of circulating lymphocytes in the PUVA-treated patients is unlikely to be a short-term consequence of the preceding exposure to treatment for several reasons. First, all blood samples were taken at least 4–7 days after the last treatment. Secondly, in a previous study (Morison *et al.*, 1979) of psoriasis patients on maintenance PUVA therapy we did not find such abnormalities. However, patients in this study were examined after only one month of maintenance treatment. Thirdly, a single erythemogenic exposure to PUVA in normal volunteers produced a decrease in the proportion of T lymphocytes but this change lasted less than 3 days and was not associated with an alteration of the response to mitogens. Thus it is likely that the decreased response to mitogens observed in the present study is a consequence of the cumulative PUVA therapy rather than an acute response to the preceding treatment. The possibility that the changes observed in PUVA-treated patients were related to the age of the patients was also considered. The PUVA-treated patients were older than the untreated patients, and also slightly older than the UV-B-treated patients. However, the changes in the PUVA-treated patients did not correlate with the age of the patients within the group. PUVA-treated patients were selected on the basis of having evidence of marked premature ageing changes in the skin as it was considered that such patients had possibly received a higher dermal dose of treatment. Presumably lymphocytes in the dermis would also receive a high exposure dose in such patients. An alternative explanation for the ageing changes of the skin and the lymphocyte abnormalities is that these patients have less capacity to repair PUVA-induced damage; however, this possibility has not been investigated. Possibly the selection of patients was biased towards patients with very severe psoriasis who may not be representative of all PUVA-treated psoriatics. Patients with severe disease which is difficult to control will tend to have the greatest exposure to the treatment. However, there are no reports of untreated patients with severe psoriasis having a marked reduction in response to mitogens, or that low levels of circulating T cells occur in patients with controlled disease. A prospective study of patients before, during and after a long course of PUVA therapy will be required to firmly establish the relationship between the treatment and alterations in lymphocytes.

Lymphocytes from the three groups of patients showed similar sensitivities to treatment with psoralen/UVA radiation *in vitro* with respect to their subsequent ability to respond to a mitogen. The mitogen responsiveness of lymphocytes obtained from patients during the early clearance phase of PUVA therapy has been reported to be more sensitive to treatment with PUVA *in vitro* than that of lymphocytes from untreated patients (Friedmann & Rogers, 1980). Our results indicate that this abnormality does not persist during prolonged PUVA therapy.

Several questions raised by our study remain unanswered. First, we have been unable to determine whether the abnormalities observed are reversible upon cessation of PUVA therapy. Cessation of therapy, in the patients in whom this has been attempted, has been promptly followed by an exacerbation of disease making it necessary to use methotrexate or PUVA to regain control of their psoriasis. Second, we attempted to determine whether the abnormalities observed in patients on PUVA therapy also occurred in patients on long-term UV-B therapy. No abnormalities were detected in these patients but the findings must be interpreted with caution. The duration of treatment was shorter and the number of exposures was smaller in the UV-B-treated patients then in the PUVA-treated patients. Further studies are needed to determine whether large cumulative doses of UV-B have effects on the immune system similar to those noted in the PUVA-treated patients. Third, the significance of our findings with respect to the immune function of patients receiving long-term PUVA therapy remains to be established. Reduced responsiveness to the contact allergen dinitrochlorobenzene has been found in PUVA-treated patients (Strauss *et al.*, 1980; Moss, Friedmann & Shuster, 1981) and thus at least one aspect of immune function is impaired in these patients. Possibly PUVA therapy may eventually result in clinical manifestations of immunosuppression such as an increased frequency of infections and neoplasia. PUVA-treated patients do not appear to be more prone to infections, other than herpes simplex, but this possibility has not been carefully evaluated. An increased incidence of skin cancer has been reported (Stern *et al.*, 1979) and it is possible that immunosuppression may be a factor in the pathogenesis of these tumours. An increased frequency of systemic neoplasia has not been observed in one long-term study of PUVA patients (Stern *et al.*, 1982). Thus far, therefore, PUVA-treated patients do not appear to be showing definite evidence of clinical manifestations of immunosuppression. However, the potential risks of immunosuppression must be included in evaluating the risk–benefit ratio involved in long and continued exposure of patients to PUVA therapy.

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Methotrexate inhibits polymorphonuclear leucocyte chemotaxis in psoriasis

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SUMMARY

Six patients suffering from widespread psoriasis were treated with 20 mg methotrexate (MTX) intramuscularly and the chemotactic activity of peripheral polymorphonuclear leucocytes (PMN) was measured before and after treatment. The modified Boyden chamber method was used and C5a, FMLP, casein and autologous fresh serum served as chemotaxins. Following MTX the chemotactic migration of PMN was inhibited by more than 50% for 2 days and slowly recovered within the following 5 days. The inhibition was present with all of the four chemotaxins used.

The results demonstrate that treatment with low dose MTX in patients with psoriasis causes a profound inhibition of PMN function. These findings support the idea that PMN play an important role in the pathogenesis of psoriasis.

The systemic administration of methotrexate (MTX) at low doses is known to be therapeutically effective in various types of psoriasis, including the pustular varieties (Weinstein & Frost, 1971, Van Scott, Auerbach & Weinstein, 1964; Roenigk, Fowler-Bergfeld & Curtis, 1969). The principal mode of action of MTX in psoriasis is thought to be based upon the inhibition of epidermal DNA synthesis by which psoriatic hyperproliferation becomes reduced (Weinstein, Goldfaden & Frost, 1971; Weinstein & Velasco, 1972). As pointed out by Weinstein & Velasco (1972), proliferating epidermal cells in psoriatic lesions are supposedly more sensitive to the effects of MTX than normal epidermal cells.

Autoradiographic studies in psoriatic epidermis after a single injection of MTX revealed the presence of 'MTX-damaged' cells together with an inhibition of mitosis lasting approximately 16 h (Weinstein, Goldfaden & Frost, 1971). Using desoxyuridine as a marker for DNA synthesis there was an absence of epidermal cell labelling after intramuscular MTX lasting again for 16 h.

The short duration of this effect makes it difficult to explain the substantial improvement of psoriasis under such treatment regimens, e.g. parenteral application of MTX once a week (Rees, 1981).

We show that low dose MTX applied intramuscularly is able substantially to inhibit

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chemotactic migration of PMN using standard chemotaxins. This may help to explain the therapeutic efficacy of MTX in psoriasis.

METHODS

Six patients were hospitalized for treatment (four female and two male, 43–83 years old, Table 1). All suffered from severe widespread psoriasis and remained untreated during the study except for the application of emollient creams. All patients had consented to the study. They received one injection of 20 mg MTX (Lederle) intramuscularly. Before and during MTX therapy renal and liver function studies as well as complete blood cell counts were performed. None of the patients had liver or renal disease and no leucocyte abnormalities including rod-shaped PMN were present.

Preparation of PMN

Blood samples were taken before any therapy was started as well as on the first, second, fourth and seventh day after the injection of MTX. PMN were isolated as previously described (Kawohl *et al.*, 1980) using a modification of the method of Henson (1971). Briefly, venous blood was drawn into 1/6 volume of acid citrate dextrose, centrifuged and the supernatant removed. The sediment was mixed with 2·5% gelatine in 0·9% NaCl and the cell suspension was allowed to sediment at 37°C for 30 min. The PMN-containing supernatant was centrifuged and contaminating erythrocytes were lysed with 0·85% NH_4Cl in distilled water. The PMN suspension was centrifuged and washed with medium TC 199 (Seromed, Munich). The final cell preparation contained more than 90% neutrophils with a viability of greater than 95% as assessed by trypan blue exclusion.

Preparation of chemotaxins

Fresh human serum was activated in the presence of 1 M ε-aminocaproic acid (EACA, Serva, Heidelberg) with Zymosan (5 mg/ml at 18°C for 30 min and 37°C for 60 min, Sigma, Munich). Chemotactic C5a was obtained by passage of activated serum (which was titrated with cold 1 M HCl to pH 4) over a Sephadex G 75 column (Pharmacia, Uppsala) with an eluant of 0·1 M acetate buffer, pH 4·6. The post-albumin fractions, which were chemotactic for PMN, were concentrated by ultrafiltration (Immersible–CX, NMWL 10000 Daltons, Millipore, Bedford, MA). The concentrated C5a fractions were adjusted to pH 3 with 0·01 N HCl. C5a was adjusted

TABLE 1. Clinical data of MTX-treated patients with widespread psoriasis

Patient no.	Age	Sex	Duration of disease (years)	Diagnosis	% skin affected
1	43	M	3	Plaque psoriasis	60
2	53	F	39	Plaque psoriasis	60
3	68	F	11	Erythrodermic psoriasis	95
4	59	M	7	Plaque psoriasis	70
5	58	F	3	Plaque psoriasis	30
6	83	F	40	Pustular psoriasis	50

just before use with TC 199, containing 0·1% human serum albumin (HSA, Behringwerke AG, Marburg) and titrated for optimal chemotaxis.

N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, Serva, Heidelberg) was dissolved in DMSO (0·01 M). FMLP was diluted to 10^{-7} M in TC 199 containing 0·1% HSA. Casein ('Hammarsten', Merck, Darmstadt, 4 mg/ml TC 199) as well as autologous fresh serum, diluted 1:2 with TC 199, served as further chemotaxins. All chemotaxins were prepared in small aliquots and stored below −70°C.

Boyden chamber chemotaxis

The chemotaxins were placed in the lower compartment (100 μl) of a modified Boyden chamber of the blind well type. The compartments of the chamber were separated by a membrane filter (cellulose nitrate filter, pore size 5·0 μm, Sartorius GmbH, Göttingen). The upper compartments were filled with 100 μl of the PMN suspension (2×10^6 cells/ml, 1% HSA in TC 199). The chambers were incubated for 2 h at 37°C. Thereafter the cells were fixed, stained and counted as previously described (Kawohl *et al.*, 1980). The chemotactic activity was expressed as the number of cells which had migrated to the bottom side of the filter. Each experiment was carried out in triplicate. Medium TC 199 containing 0·1% HSA was used as a negative control.

RESULTS

The mean value of PMN chemotactic activity of all six patients before MTX therapy served as baseline value (100%). Chemotactic activity was determined 1, 2, 4 and 7 days after the injection of MTX.

Using C5a (Fig. 1a) as well as FMLP (Fig. 1b), both of which are known to be strong chemotaxins, a significant inhibition of chemotactic activity can be seen. For both chemotaxins this inhibition amounts to nearly 50%. It begins within the first day after the injection of MTX and remains at this level throughout the second day. Recovery slowly takes place on the fourth day and by the seventh day after MTX the inhibition of PMN chemotaxis is less than 15% (Fig. 1a,b).

Using casein and autologous fresh serum (Table 2) as chemo-attractants similar results were obtained. On the first day after MTX injection the inhibition of chemotactic migration was nearly 50%. Recovery began on the following day, and on the fourth day after MTX the inhibition was near 30%. Medium TC 199 containing 0·1% HSA, used as a negative control, showed no evidence of being chemotactic for PMN.

DISCUSSION

Methotrexate is known as a potent inhibitor of dihydrofolate reductase, whereby cellular DNA synthesis is reduced or blocked. This effect is seen in a varity of benign as well as neoplastic tissues and is generally considered the predominant mode of action of MTX in producing cytotoxicity.

In psoriatic skin MTX is thought to inhibit epidermal DNA synthesis thus reducing psoriatic hyperproliferation (Weinstein, Goldfaden & Frost, 1971). The comparatively low dose of MTX (20–25 mg intramuscularly once weekly) which is sufficient to cause clearing of the affected skin is explained by a higher sensitivity of psoriatic epidermal cells for MTX (Weinstein, Goldfaden & Frost, 1971; Newburger, Weinstein & McCullough, 1978; Weinstein & McCullough, 1976).

On the other hand, the lack of effectiveness of MTX when applied topically remains

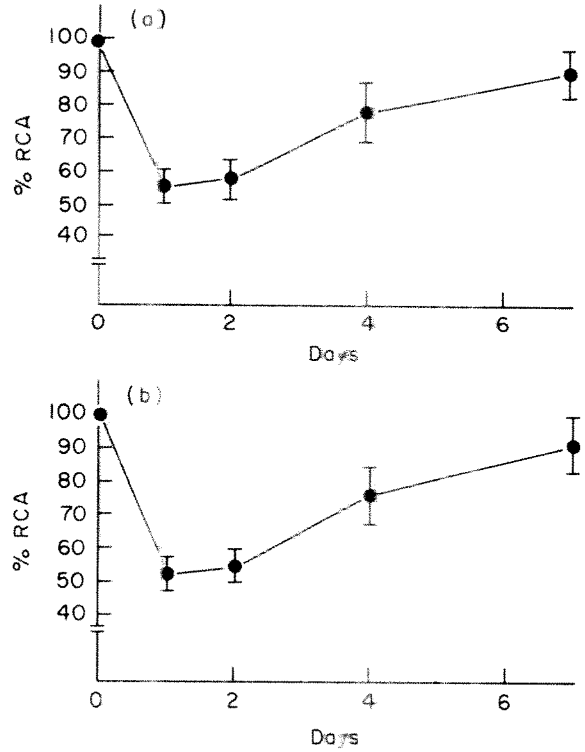

FIGURE 1. Effect of a single injection of MTX (20 mg i.m.) on PMN chemotaxis, using C5a (a) and FMLP (b) as chemotaxin. The specimens were obtained before and 1,2,4 and 7 days after MTX injection. Each point represents the mean value obtained from six patients. The vertical lines represent the range of values. RCA = relative chemotactic activity.

unexplained (Stewart, Wallace & Runikis, 1972; Comaish & Juhlin, 1969). In addition, the short duration of the inhibitory effects of MTX on epidermal cell proliferation (e.g. 16 h, Weinstein, Goldfaden & Frost, 1971) makes it difficult to understand the time-scale of the therapeutic efficacy of this drug.

The results presented here demonstrate a profound depression of PMN migration using

TABLE 2. Chemotactic activity of PMN following 20 mg MTX using casein and autologous serum as chemotaxins*

	Chemotactic activity (per cent of control)			
	Days after MTX			
Chemotaxin	1	2	4	7
Casein (4 mg/ml)	58·4 ± 4·2	57·4 ± 5·3	74·2 ± 9·2	87·2 ± 7·7
Diluted autologous serum (1:2)	53·8 ± 3·9	55·5 ± 4·8	76·2 ± 7·4	85·9 ± 5·9

* Data obtained one day before MTX treatment were taken as 100%.

various chemotaxins. This was constantly present in all of the patients studied. The extent and the duration of PMN inhibition after MTX were similar despite the variability of the clinical picture of psoriasis. This chemotactic depression is seen within the first 24 h after MTX and persists on the following day, whereas nearly normal PMN migration is present after day 7.

Since dihydrofolate reductase is not detectable in normal granulocytes (Bertino *et al.*, 1962) it seems improbable that the effect of MTX is based upon folic acid antagonism within these cells. Also, impairment of the production of leucocytes in the bone marrow by MTX appears unlikely since the white blood count during MTX therapy remained unchanged (unpublished data).

Moreover, Dörmer *et al.*, (1981) applied high dose methotrexate (2·0 and 7·5 g MTX per square metre body surface followed by leucovorin rescue) and determined the relative production rates of PMN. Compared with erythrocytes there was only a minor decrease of the relative granulopoietic cell production 2 h after MTX which was followed by an overshoot reaction after 48 h. This is additional evidence that other explanations for the effect observed here need to be considered.

It is of interest that the inhibition was present with all chemotaxins used including C5a and FMLP. Since the latter compounds are known to react with separate membrane receptors (Chenoweth & Hugli, 1978; Williams *et al.*, 1977), receptor binding can be ruled out. More centrally located alterations, e.g. interference of MTX with the contractile apparatus, could play a role.

Using the Boyden chamber system, Cream & Pole (1980) recently found that MTX added to PMN *in vitro* inhibited PMN chemotaxis against casein by 25–45%. The concentrations of MTX used by these authors (1·25–20 μg per ml) are higher than the upper serum levels to be expected in patients treated with 20 mg intramuscularly (Halprin, Fukui & Okhawara, 1971). The apparent discrepancy between the *in vitro* findings and our *in vivo* effects cannot be fully explained at the moment.

Since PMN in patients with psoriasis are reported to be chemotactically hyper-responsive (Wahba *et al.*, 1978; Langner *et al.*, 1978; Kawohl *et al.*, 1980; Michaelsson, 1980) it cannot be ruled out that the effects of MTX seen here are related to this hyperreactivity. Our findings nevertheless point towards the important role which PMN seem to play in psoriasis. Indeed the prompt involution of skin lesions after low dose MTX in pustular as well as in vulgaris type psoriasis may be caused by the transient immobilization of PMN. The duration of this effect coincides with the weekly treatment generally used (Bergstresser & McKenzie, 1976).

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Pharmacology and Treatment

Dithranol–UV-A phototherapy (DUVA) for psoriasis: a treatment without dressings

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SUMMARY

Dithranol in yellow soft paraffin with 2% salicylic acid was applied daily (increasing concentrations from 0·2% to 5%) for 1 hour to twenty consecutive patients with diffuse psoriasis. The ointment was then washed off and patients were irradiated with 10 J cm^{-2} of UV-A radiation since the absorption spectrum of dithranol is maximum in the UV-A waveband. The median clearance time was 14 days (range 7–39 days). No dressings were required. The patients were allowed to apply an emollient if the skin became dry.

The Ingram regimen (Ingram, 1953) which involves tar baths, ultraviolet radiation (UVR) and applications of dithranol, has been the mainstay of dithranol therapy of psoriasis for over 20 years. Dithranol is unpleasant and stains clothing, and overall dressings are required. The most common source of UVR used in this regimen is the medium pressure mercury vapour lamp and the spectral power distribution of this source is such that those wavelengths in the UV-B region (280–315 nm) are probably the major therapeutic components of the emission spectrum (Bowers *et al.*, 1966).

Dithranol undergoes light catalyzed oxidation and Raab (1981) states that UV-irradiated dithranol exhibits greater activity than unirradiated dithranol but gives no indication of the wavelengths primarily responsible for this increased activity. We decided, therefore, to measure the absorption spectrum of both irradiated and unirradiated dithranol with a view to exploiting its possible photochemical properties in the treatment of psoriasis.

METHODS

Dithranol powder was dissolved in chloroform at a concentration of 0·02% by weight. The absorption spectrum was measured in the spectral interval 300–450 nm and was found to peak at around 360 nm. The solution was then irradiated with UV-A obtained from a xenon arc lamp in

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conjunction with appropriate optical filters (Schott WG320 and UG1). Following irradiation the solution exhibited a yellowish tinge as was evident from the increase in the absorption spectrum in the blue region (400–500 nm) of the visible spectrum (see Fig. 1). The absorption spectra of irradiated and unirradiated dithranol are compared in Fig. 1 with the spectral power distribution of the UV-A fluorescent lamps commonly employed in PUVA irradiation units (Diffey, 1982). It can be seen that there is a fortuitous close agreement between the emission spectrum of lamps and the absorption spectrum of dithranol, which implies that the radiation emitted by the lamps will be efficiently absorbed by the dithranol resulting in a near maximum therapeutic efficiency.

A pilot study of the photosensitizing potential of dithranol in normal (non-psoriatic) skin was carried out as follows:

Dithranol at three different concentrations (1%, 0·1% and 0·02%) in salicylic acid and vaseline was applied with cotton wool balls to the back of a white male. After 30 min the preparation was removed from each skin site with Savlon® and the sites were irradiated with UV-A by means of an irradiation monochromator (wavelength range 320–380 nm, peaking at 350 nm). Each of the three sites was given doses of 1, 2, 5, 7 and 10 J cm^{-2}. In addition a control skin site with no dithranol applied was irradiated at doses up to 40 J cm^{-2}. The sites were observed at 24 and 48 h post-irradiation and in no case was any erythema or abnormal morphological lesion observed. The minimal erythema dose on the unsensitized skin was 40 J cm^{-2}. We believe, therefore, it would be safe to give 10 J cm^{-2} to all patients at every treatment without risk of burning.

Runne & Kunze (1982) have achieved a mean clearance time of 26·9 days (range 12–46 days) using high concentrations of dithranol applied for short periods and Schalla *et al.* (1981) have shown that penetration kinetics of dithranol are accelerated in lesional skin so that washing off dithranol removes much more from normal than lesional skin. Selim *et al.* (1981) further showed that most of the topically applied dithranol has entered the epidermis within 1 hour of application.

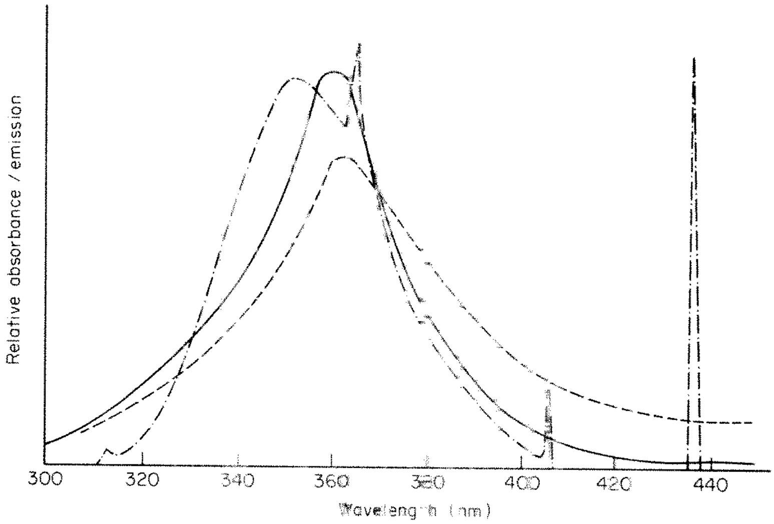

FIGURE 1. The absorption spectrum of unirradiated (——) and irradiated (– – –) dithranol, and the spectral emission of a UV-A fluorescent lamp (— · —).

The object of this study was to investigate the efficacy of adding UV-A to the Runne and Kunze regimen. Since the absorption spectrum of dithranol lies mainly in this spectral region it was presumed that UV-A would maximize the activity of dithranol in the skin.

Twenty Caucasians with generalized psoriasis (eight males, age range 11–61 years; twelve females, age range 19–78 years) were admitted consecutively to the study. In each case more than 15% of the body surface was affected by psoriasis and the patients would normally have been treated by the Ingram regimen in our department.

Dithranol in yellow soft paraffin with 2% salicylic acid was applied and left on the skin for 1 hour during which time patients wore a theatre gown for privacy. The dithranol was then washed off in a bath containing detergent (Teepol®, Shell) and Oilatum® emollient (Stiefel). The initial concentration of dithranol was 0·1% which was increased according to clinical response up to 5% over the treatment period of usually about 14 days. Immediately after bathing the patients received 10 J cm^{-2} to each side of the body in a semi-cylindrical UV-A apparatus (Canterbury Instruments) containing 24 Philips TL85 100 W/09 lamps, 180 cm in length.

Scalp psoriasis was treated with preparation of dithranol and urea (Hindson, 1980) (Psoradrate®; Eaton Laboratories). Lesions in the intertriginous areas were treated with hydrocortisone 17-butyrate (Locoid® ointment, Brocades GB Ltd.), although DUVA therapy was effective for lesions in the antecubital fossae.

Treatments were given once daily on consecutive days until the psoriasis cleared. Patients who complained of dryness of the lesions were allowed to apply an emollient (Unguentum Merck®, Merck, Sharp & Dohme).

RESULTS

The median time to healing was 14 days with a range of 7–39 days. All patients in this study responded to DUVA therapy and none had to be withdrawn. One patient had diffuse vitiligo of the trunk where there were also psoriatic plaques, but did not burn. One patient who had been withdrawn from methotrexate therapy because of liver damage cleared with DUVA therapy but experienced a moderate erythema on her shins. These lesions were treated with a topical steroid and occluded from UV-A. One patient cleared who had in past years been unable to tolerate the Ingram regimen because of burning. One patient with generalized psoriasis and facial lesions tolerated DUVA therapy using 0·1% dithranol on the face and cleared. Finally one patient discharged herself from the trial because there were not dressings which she had been used to receiving previously. Lesional staining was much less marked than with other forms of dithranol therapy, and all patients acquired marked tanning of normal skin.

DISCUSSION

Dithranol has formed one component of therapeutic regimen for psoriasis for many years (Ingram, 1953). More recently, it has been shown that UV-A alone in large doses also leads to improvement of psoriasis (Parrish, 1977), but since the daily exposure dose of UV-A must be 30–300 J cm^{-2}, the lack of light sources of adequate intensity makes treatment of large areas impractical. By utilizing the photosensitizing potential of dithranol with UV-A radiation, we believe we have developed a regimen which does not involve dressings, makes whole body treatment feasible, and results in a median clearing time of about 14 days, a time which compares favourably with that generally quoted for the Ingram regimen (Rogers *et al.*, 1979). Washing off dithranol after a short period decreases the possibility of burning normal skin and enables flexural and facial lesions to be treated.

One advantage of this treatment is a saving of nursing time. It takes one trained nurse on her own in our department 25 min to apply dithranol in Lassars paste with an all-over dressing for the Ingram regimen, compared with 10 min to apply dithranol in Vaseline for the DUVA regimen. Patients were able to wash off dithranol in a warm bath with Teepol® and soap and water if they wished. Supervision or help was not necessary other than for patients with disabilities such as arthritis. Patients previously intolerant of the Ingram regimen have tolerated DUVA therapy. Furthermore the absence of dressings for long periods makes DUVA therapy personally less intrusive and cosmetically more acceptable.

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We are grateful to Dr B. Gordon, Consultant Dermatologist, who allowed us to treat four of his patients.

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Thalidomide in the treatment of sixty cases of chronic discoid lupus erythematosus

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SUMMARY

The therapeutic effect of thalidomide in chronic discoid lupus erythematosus (CDLE) was studied in sixty patients who were followed up for 2 years. In fifty-four patients (90%) a complete or marked regression of the disease was observed, but when the thalidomide was stopped, thirty out of forty-one (71%) patients relapsed. Patients undergoing a second course of thalidomide treatment again responded well. Nine of the patients in whom the disease recurred after successful treatment with thalidomide and who had been unresponsive to intermittent treatment with antimalarials, showed a good response to a second or third course with thalidomide. Mild side-effects were common and 25% of patients complained of slight to moderate polyneuritic symptoms. Since electroneurological examinations had not been performed before the thalidomide therapy, the frequency of neurological side-effects cannot be accurately calculated but we recommend neurological examinations before and periodically during thalidomide treatment.

Thalidomide is a very effective drug in CDLE, but in most cases it exerts its effect only whilst treatment is continued. Its use should be restricted to patients resistant to topical steroids and systemic antimalarials.

Following the reports of Barba-Rubio & Franco-Gonzales (1975) and Samsoen, Grosshans & Basset (1980) we have confirmed a good response to thalidomide in twenty-four patients suffering from chronic discoid lupus erythematosus (CDLE) (Knop *et al.*, 1981). These patients have been followed up and we have since increased the number of patients treated with thalidomide to sixty. We now report the results of our extended study.

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PATIENTS AND METHODS

All patients in this study were under regular observation and treatment in our outpatient clinic. Specific previous medication was withdrawn at least one month before starting thalidomide therapy, and no other treatment (with the exception of light protection) was allowed. Because of the teratogenic effect of thalidomide female patients were informed about the possible risks associated with this drug and were only accepted for this trial after consultation with a gynaecologist to rule out pregnancy and to ensure adequate contraceptive measures. Because of the sedative side-effects of the drug the patients were advised not to drive, or handle machinery. Clinical data of the patients are given in Table 1. All had a typical history and clinical features compatible with a diagnosis of CDLE and this was confirmed by histology and by positive direct immunofluorescence. Most patients had been unsuccessfully treated with topical steroids and oral antimalarials; in a few cases azathioprine had been used. The mean duration of the disease was 8·4 years (range:0·6–34). In most patients the lesions were on the head and neck, but others had widespread lesions on the arms, trunk and back. Photographs were taken before, during and after therapy with thalidomide.

TABLE 1. Clinical data of patients studied

	No.
Number of patients	
Female	45
Male	15
Lesions localized (to head and neck)	41
Lesions disseminated	19
Previous treatment	
Glucocorticoids, topical	53
Glucocorticoids, systemic	17
Antimalarials	46
Azathioprine	7

The patients were seen at 3- to 4-week intervals and laboratory studies included white blood count, red blood count, haemoglobin levels, platelet counts, transaminases, serum creatinine, erythrocyte sedimentation, serum immunoglobulins (IgG, IgM, IgA), C3, C4, anti-nuclear antibodies and anti-nDNA. Patients complaining of paraesthesia and/or sensory loss were seen by a neurologist to exclude other causes of peripheral neuropathy. The motor nerve conduction velocity of the right median nerve was determined in all such cases. In some cases the sensory nerve conduction velocity was measured by computerized evaluation of the evoked summation potentials.

The dosage of thalidomide (Grünenthal Co., Stolberg, FRG) was similar to that reported earlier (Knop *et al.*, 1981). Initially patients were given a daily dose of 400 mg (in two equally divided doses, at 1200 h and 2200 h). Following a marked clinical response the dosage was reduced every month until a maintenance dosage of 50–100 mg a day was reached. This dosage was maintained for 3 to 5 months or longer. The response to thalidomide has been evaluated in patients who had either completed a course of treatment with thalidomide or who had been under treatment for more than 3 months.

RESULTS

Table 2 shows the results after thalidomide treatment. Ninety per cent of the patients showed a favourable response and in 65% of the total a complete regression was observed. As described earlier, there was a somewhat better response in females but males also showed a marked regression of lesions.

TABLE 2. Response to thalidomide treatment in sixty patients

	Female	Male	Total
Signs of regression, but no substantial improvement	3 (7%)	3 (20%)	6 (10%)
Significant regression with some residual activity	8 (18%)	7 (47%)	15 (25%)
Complete regression (occasional minimal activity)	34 (75%)	5 (33%)	39 (65%)

TABLE 3. Relapse rate in forty-one patients followed-up after discontinuation of thalidomide therapy

	No. of patients	Mean and range of disease-free interval (months)
No relapse	11 (27%)	5 (2–8)
Relapse	*30 (73%)	3·5 (0·5–12)

* Sixteen of the relapsing patients were treated again with thalidomide and all sixteen then showed a good response.

Table 3 shows the frequency of relapses after cessation of therapy. Eleven (27%) of forty-one patients completing a course of thalidomide suffered no relapse in a subsequent 2–8 months; in thirty patients a relapse occurred following cessation of therapy. In all cases where a further course of treatment with thalidomide was given the lesions showed a good response. Nine patients who relapsed after successful therapy with thalidomide and who were unresponsive to an intermittent course of antimalarials, experienced a good response when given a second or third course of thalidomide. In some patients a reduction of the dosage resulted in a flare-up of lesions but this could be controlled by increasing the dose once more (Table 4).

Although there were frequent side-effects, most were not serious (Table 5). Fifteen patients complained of sensory disturbances during thalidomide treatment (Table 6). Most patients complained of paraesthesiae and/or sensory loss, usually of the distal extremities. Neuropathy was classified and subdivided according to clinical and electrophysiological criteria into four

TABLE 4. Effect of low dose maintenance therapy (50–100 mg daily) in twenty patients

	No. of patients	Mean and range of disease-free interval (months) during low dose therapy
No relapse	13 (65%)	7 (1–16)
Relapse	*7 (35%)	5 (1–9)

* All seven patients became free of lesions after increasing the dose.

TABLE 5. Side-effects of thalidomide therapy (laboratory results were unchanged)

	No. of patients affected	No. not tolerating therapy
Somnolence	60	1
Constipation	19	—
Circulatory disturbances	7	3
Oral dryness	2	—
Rash	7	1
Oedema (lower leg)	4	1
Polyneuritic symptoms	15	15

TABLE 6. Electro-neurological analysis in fifteen patients who complained of paraesthesiae or sensory loss

Group	Classification of neuropathy	No. of patients affected (%)
1	No peripheral neuropathy	3 (20)
2	Slight form of mainly sensory peripheral neuropathy	8 (53)
3	Moderate sensory peripheral neuropathy with slight motor lesions	3 (20)
4	Severe peripheral neuropathy	1 (7)

groups (*see* Table 6). The clinical and electrophysiological examinations revealed no peripheral neuropathy in three out of fifteen patients (20%, group 1), slight sensory disturbances in eight (53%, group 2), moderate lesions in three (20%, group 3) and severe peripheral neuropathy in one (7%, group 4). In three patients (two in group 2, one in group 3) alcohol abuse could not be excluded. The mean total thalidomide intake of the patients affected by polyneuropathy was 85 g (range 15·5–132 g).

DISCUSSION

This extended follow-up study confirms our previous results showing effective control of CDLE lesions with thalidomide (Knop *et al.*, 1981). Our results are slightly better than those of Samsoen *et al.* (1980) but this may be due to the higher initial dosage used. After cessation of thalidomide therapy the disease relapsed in many cases. However, most of the lesions were not as severe as they were before thalidomide treatment. In cases where thalidomide was restarted a good response was observed and this suggests that thalidomide probably suppresses disease activity only as long as it is administered. In this respect thalidomide appears similar to the antimalarials, although it is much more effective.

Most of the side-effects observed in this study have been previously described (Knop *et al.*, 1981); Sheskin *et al.*, 1975; Samsoen *et al.*, 1980). Almost all the side-effects disappeared after withdrawal of the drug, with the exception of sensory disturbances due to neuropathy which had not been noticed in our previous study. In many cases the symptoms of neuropathy disappeared soon after drug withdrawal but in a few patients, who were affected more severely, paraesthesiae or sensory loss was still present one year after the end of treatment, although some improvement was noticed. Neuropathy due to toxic effects of thalidomide has been described by several authors in patients who have used this drug as a sedative (for review *see* Gibbels, 1967). Some authors reported full recovery in 25–35% of patients and partial recovery in the rest (Amelung & Püntmann, 1966; Fullerton & O'Sullivan, 1968; Gibbels, 1967). The high incidence of peripheral neuropathy, although trivial in most cases, was unexpected particularly because polyneuropathy does not seem to develop in the treatment of erythema nodosum leprosum with thalidomide (Sheskin & Yaar, 1979). This discrepancy cannot simply be explained by the total amount of thalidomide taken or the duration of treatment since the dosage scheme used by us was similar to that as described by Sheskin (1975). Other authors, using lower doses of thalidomide for treatment of CDLE have not reported polyneuropathy as a major problem (Samsoen *et al.*, 1980; Barba-Rubio & Franco-Gonzales, 1975). The true incidence of polyneuropathy due to thalidomide cannot be evaluated by this study because detailed neurological examinations had not been performed before starting thalidomide therapy. Clinical and electrophysiological neurological examinations are now performed before and during thalidomide treatment in order to assess the true incidence of polyneuropathy caused by thalidomide and to minimize its risk. The doses of thalidomide used, which were similar to those used for treatment of lepra reactions (Sheskin, 1975) may have been too high. For the long-term treatment of CDLE lower doses should now be tried. It should be mentioned, however, that in our experience many lesions could be cured only by a high initial dosage (400 mg/day).

We conclude from this study that thalidomide should be used in patients with severe CDLE unresponsive to topical steroids and systemic antimalarials. Lower initial and maintenance doses such as those reported by Samsoen *et al.* (1980) should be tried and patients should be under close neurological supervision. A neurological assessment should be performed before, during and after thalidomide treatment. Alcohol and potentially neurotoxic drugs should be avoided during thalidomide therapy as they may accentuate thalidomide toxicity (Amelung & Püntmann, 1966).

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Thalidomide in actinic prurigo

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SUMMARY

Fourteen patients suffering from actinic prurigo were treated with thalidomide. Eleven patients showed lasting improvement on the drug and three of these remained symptom-free after discontinuing therapy. No major side-effects were observed. Thalidomide is an effective drug in the treatment of actinic prurigo but it must be used with adequate contraception in women of child-bearing age.

Thalidomide, a derivative of glutamic acid, was originally introduced as a hypnotic agent (Somers, 1960). It achieved widespread use because of its apparent low toxicity and its selective action in the absence of respiratory depressant or narcotic effect. Unfortunately, the drug proved to be teratogenic, and for this reason was withdrawn from general use (Lancet, 1962). Other major side-effects of the drug include peripheral neuropathy (Fullerton & Kremer, 1961) and myxoedema (Alexander, 1961).

Thalidomide has an immunosuppressive action and has proved of value in the treatment of the lepra reaction (Sheskin, 1965; Waters, 1971). Londoño (1973) was the first to use thalidomide in actinic prurigo (Hutchinson's summer prurigo), believing this condition to have a similar immunological mechanism. He treated thirty-four patients, using an adult starting dose of 300 mg daily, maintaining this high dose until improvement was noted, and then reducing progressively to the minimum effective dosage. There was noticeable improvement of lesions in thirty-two of the thirty-four patients although the time interval between onset of therapy and clinical remission was considerably longer than in the lepra reaction. Slight response was noted after an average of 26 days but several patients continued to improve up to 90 days after initiating therapy. Relapse occurred in all patients who discontinued therapy (Londoño, 1973).

Flores (1975) described similarly encouraging results in the treatment of 'prurigo solar de altiplanicie', a condition that is morphologically identical to actinic prurigo. This condition is readily distinguishable from polymorphic light eruption as it is understood in the United Kingdom (Meara *et al.*, 1971; Magnus, 1976; Calnan & Meara, 1977; Scheen, Connolly & Dicken, 1981), but it is included in the term 'polymorphous light eruption' by many authors, e.g. Epstein, 1966; Brodthagen, 1969; Harber & Bickers, 1981. Saul, Flores & Novales (1976)

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TABLE 1. Patient details

Initials	Sex	Age	History	Physical findings	Monochromator responses	Thalidomide dosage, apparent adverse side-effects and outcome
M.B.	F	35	7 years itchy rash on face. Mild itching of upper back	Papular excoriations on cheeks, back, upper limbs and scalp	Not done	100 mg daily. Clear after 2–3 weeks. Noted drowsiness and lack of energy. Relapsed after stopping the drug. Controlled on 200 mg/week for 1 year. Now off thalidomide. Occasional erythema only
A.B.	F	20	11 years widespread itchy rash on face, neck and limbs. Rash not obviously worsened by sunlight	Excoriations on cheeks, back of neck, forearms dorsa of hands, base of spine and lower legs	Abnormal (300–360 nm)	200 mg daily. Initial worsening. Considerable improvement after 1 week. Noted drowsiness. Well controlled on 100 mg/day for 3 years. Rash then recurred and persisted despite increase to 300 mg/day. Drug discontinued. Rash now well controlled on mepacrine 100 mg/day
A.M.	M	15	13 years widespread itchy rash. Mother and sister also affected. Mother's rash cleared spontaneously at puberty	Excoriated papules face, upper arms and buttocks	Not done	100 mg for 3 weeks. Clear after 1 week. No side-effects. Off treatment. No recurrence in subsequent 2 months
D.R.	F	52	4 years of irritating 'blisters' on skin, leading to persistent itchy lumps. Not restricted to light-exposed sites	Nodular pruriginous areas on arms, wrists and shins. Scaling, weeping areas on scalp and face	Abnormal (300–340 nm)	100 mg for 2 weeks. Considerable improvement after 2 weeks. Complained of feeling 'disembodied', Indigestion. Rash recurred after 1 month off treatment. Not controlled on 100 mg *alt die* and 100 mg daily produced intolerable drowsiness and dizziness
E.P.	F	8	2 years very itchy rash on face, arms and legs. No direct relationship to sun	Eczematous pruriginous eruption on cheeks, distal arms and legs. Papules on sacrum sparing of forehead	Abnormal (300–320 nm)	50 mg daily. Improved initially but slight relapse despite increasing dose. No side-effects. Controlled except for mild facial eruption. 100 mg every 3–4 days
V.B.	F	36	3 years intensely itchy rash on face, arms and legs. Worse in summer	Papules, excoriations and scars on hands, neck, sacrum and lower legs	No definite abnormality	100 mg for 3 months. No effect for 2 weeks, then considerable improvement. Relapsed one week after stopping drug. Noted coarse hairs on backs of hands and transient paraesthesiae of left arm and leg. Controlled on 100 mg *alt die* 150 mg for 2 weeks.
J.J.	F	48	18 months itchy papular rash on cheeks, nose and forehead	Excoriated lesions on upper arms, V of neck	Not done	Considerable improvement after 1 week. No adverse side-effects. Controlled on 100 mg daily

G.Mc.H	F	12	3 years itchy rash on face, arms, legs and shoulders	Excoriated papules on face, hands, upper arms and upper legs	Abnormal (300–320 nm)	100 mg for 3 months. Considerable improvement after 2 weeks. No adverse side-effects. Relapsed 1–2 weeks after. stopping drug. Controlled on 100 mg daily
D.McL	M	13	2–3 years itchy rash on face. Worse in summer	Excoriations on upper arms, forearms and cheeks	Abnormal (300–320 nm)	50 mg for 2 months. Improvement within 1 week. No adverse side-effects. Relapsed 1–2 weeks after stopping drug. Controlled on 50–100 mg weekly
J.M.	F	33	7 years itchy rash on legs, backs of hands, cheeks and nose. Swelling of cheeks. Worse in summer	Erythematous papular eruption on cheeks, backs of hands, extensor surfaces of arms to elbows, shins, calves and dorsa of feet	Abnormal (300–360 nm)	200 mg for 4 days. Developed headache and morbilliform rash after 2–3 days. Treatment stopped and reintroduced gradually at doses of 25/50/100/200 mg *alt die*. Maintained on 200 mg *alt die*. Considerable improvement after 2 weeks. No further adverse side-effects. Controlled on 200 mg *alt die*.
J.W.	F	42	6 years itchy rash on cheeks, upper chest and upper back. Worse in summer	Excoriations on cheeks. Prurigo nodules on upper chest and back	Abnormal (300 nm)	100 mg daily. Slight improvement over 2 weeks. 200 mg daily. Clear after 2 months. No side-effects. Controlled on 100 mg every 5–6 days
A.McL	M	5	8 months red itchy lumps on cheeks, bridge of nose and backs of hands. Worse in summer but not directly aggravated by sunlight	Excoriated papules on face and dorsa of hands and buttocks	Abnormal (300 nm)	100 mg daily. Cleared after 3/52. No side-effects. Clear when last seen and off therapy for 1 month
M.K.	F	47	3 years itching of light-exposed areas 1 day after sun exposure. Swelling and rash appear on exposed sites 2–7 days after	Excoriated papules on face and forearms	Not done	100 mg daily. Cleared after 1–2 weeks. No side-effects. Controlled on 100 mg *alt die*
A.C.	F	54	Since age of 18 itchy pinpoint papules 1–2 hours after exposure to sunlight. Clear after 2–3 days. Since aged 30, itchy blistering eruption on cheeks, forearms and lower legs. Persists throughout year	Excoriated papules on lower limbs and buttocks. Scaling maculopapular rash on cheeks	Abnormal (300–340 nm)	100 mg daily produced dizziness after 3 days. 50 mg produced drowsiness and after 4 days itchy papular rash appeared on thighs, improving after thalidomide stopped. Not reintroduced. No benefit noted while on drug

treated twenty-five patients with 'polymorphous light eruption' with thalidomide in a dose of 100–200 mg/day. Improvement was observed in 88% of patients after an average of 15 days. 56% of patients had complete clearance of lesions and of pruritus. The drug was discontinued in twenty (80%) and twelve (50%) remained asymptomatic 1 year after discontinuing therapy, in contrast to the findings of Londoño (1973). The only side-effects noted were slight somnolence and increased appetite (Saul *et al.*, 1976).

To date there are no European reports of the use of thalidomide in actinic prurigo, with the exception of two case reports in the paper by Calnan & Meara (1977).

PATIENTS AND METHODS

Our experience with thalidomide in actinic prurigo is detailed in Table 1. Fourteen patients were treated with the drug. All had classical features of actinic prurigo, namely erythematous, excoriated persistent patches on light-exposed areas, particularly on the cheeks and distal half of the nose, but also in some patients involving covered areas such as the buttocks (Calnan & Meara, 1977). However, two patients (M.K. and A.C.) gave histories strongly suggestive of additional polymorphic light eruption, with sheeted or papular erythema occurring within hours of sun exposure. Although a number of our patients were children, onset of the disease also occurred in adult life, even extending to middle age in some patients, giving an age range of 4–48 years. Females predominated (11 : 3). Initial daily dose of thalidomide was 50–100 mg for children and 100–200 mg for adults.

RESULTS

Thirteen out of fourteen patients gained initial benefit from the drug. The remaining patient was unable to tolerate a sufficient dosage because of dizziness. Eleven patients maintained considerable improvement and three patients have remained symptom-free after discontinuing the drug completely. The other eight patients required maintenance therapy of between 50–100 mg weekly and 100 mg daily. Two patients relapsed on therapy, despite initial improvement; one (A.B.) is now well controlled on mepacrine.

No major or irreversible adverse side-effects have been observed in our patients who have received thalidomide. However, minor side-effects were not infrequent. Somnolence, as expected, is a relatively common feature and for this reason the drug should be taken at night. Three of our patients noted drowsiness and lassitude. Two patients described dizziness and a 'disembodied feeling'; in one patient (A.C.), mentioned above, this was severe enough to prevent an adequate trial of the therapy. A morbilliform rash developed in two patients, although in one patient (J.M.), the rash did not reappear after reintroduction of the drug on a lower dose. Transient paraesthesiae were described by one patient but a true peripheral neuropathy did not develop.

In addition to the patients shown in Table 1, thalidomide was given to four patients with actinic reticuloid but without any benefit.

DISCUSSION

Thalidomide has been used in a variety of dermatological conditions with apparent benefit, including Weber–Christian disease (Eravelly & Waters, 1977), recurrent aphthae (Mascaro, Lecha & Torras, 1979), subacute cutaneous lupus erythematosus (Naafs *et al.*, 1982) and discoid lupus erythematosus (Levi, Saggiorato & Crippa, 1980; Knop *et al.*, 1983). The drug appears to have a specific action in actinic prurigo, a condition of unknown pathogenesis, which is resistant to other available forms of treatment. In our experience, thalidomide has been relatively free of side-effects, well-tolerated and effective in the management of this condition. However, it should be remembered that the peripheral neuropathy occasionally related to the drug can be devastating and irreversible (Fullerton & Kremer, 1961). We recommend a starting dose of 50 mg in children and 100 mg in adults with a maximum dose of no more than 200 mg daily. If minor side-effects develop, therapy should be discontinued and reintroduced cautiously, e.g. 50 mg *alt die*, increasing gradually to the minimum effective therapeutic dose. Once the disease is controlled, an attempt should be made to reduce or even discontinue the therapy. Because of its teratogenicity, thalidomide should be used with adequate contraception in any female of child-bearing age.

Further studies are needed into the pathogenesis of actinic prurigo and the mode of action of thalidomide in this condition.

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Case Reports

Dowling-Degos disease (reticulate pigmented anomaly of the flexures) is an autosomal dominant condition

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SUMMARY

A family is described in which eleven members were affected by the Dowling-Degos disease, which appeared to be transmitted as an autosomal dominant.

Dowling-Degos disease (DDD) is a rare genodermatosis characterized by a spotted and reticulate pigmentation of the flexures. Twenty cases have so far been described (Wilson-Jones & Grice, 1978; Howell & Freeman, 1978; Grosshans *et al.*, 1980). The disease may affect more than one member of a family but neither a complete pedigree nor the mode of transmission have been reported previously.

We now report two cases of DDD from a large family in which an autosomal dominant transmission was apparent in eleven affected members.

CASE REPORT

Case 1. A 45-year-old Sicilian woman noticed a rapidly increasing and persistent pigmentation of her axillae and other flexures soon after puberty. On examination all the flexures and the neck showed a reticulate dotted brownish pigmentation (Fig. 1). Perioral pitted scars and dark comedo-like lesions were also visible on the upper eyelids. A biopsy from the macular lesion of the axilla revealed the typical delicate pigmented epithelial downgrowths of DDD (Fig. 2).

Case 2 was a 67-year-old woman, the mother of patient 1, who noted the onset of her disease a few years after the menarche. On examination her skin showed features almost identical to those of her daughter (Fig. 3). Biopsy was not performed.

The family history allowed the pedigree to be constructed (Fig. 4).

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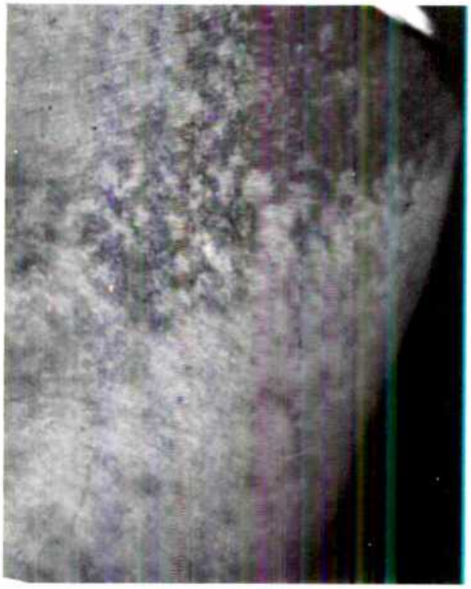

FIGURE 1. Dowling-Degos disease. Case 1, showing characteristic pigmentation.

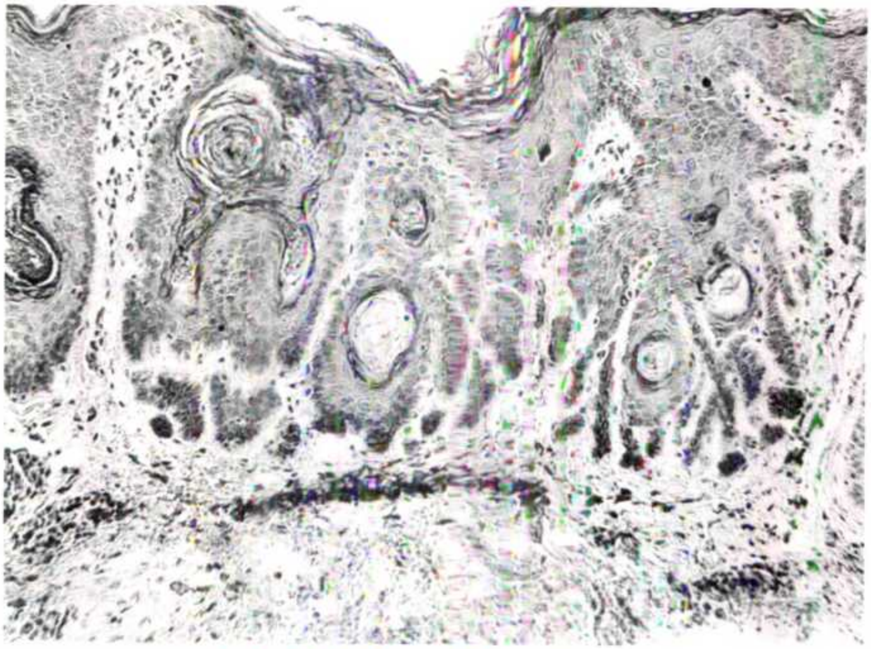

FIGURE 2. Histology of axillary skin from Case 1.

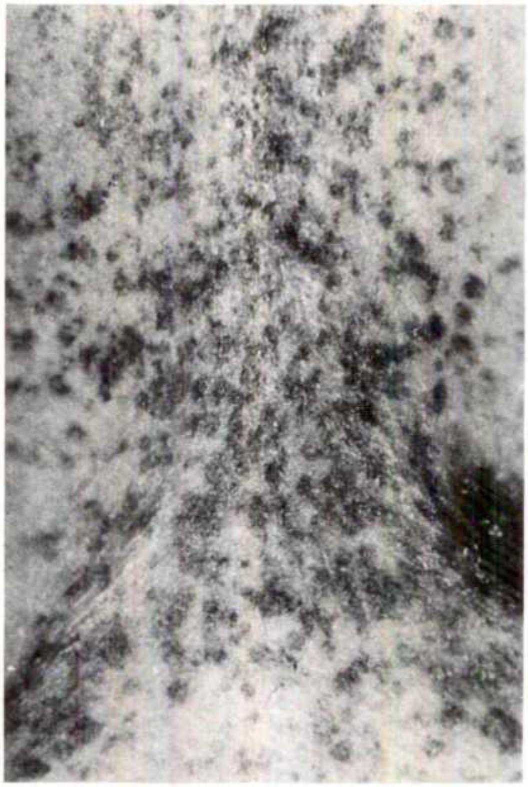

FIGURE 3. Dowling-Degos disease. Case 2, showing stippled pigmentation.

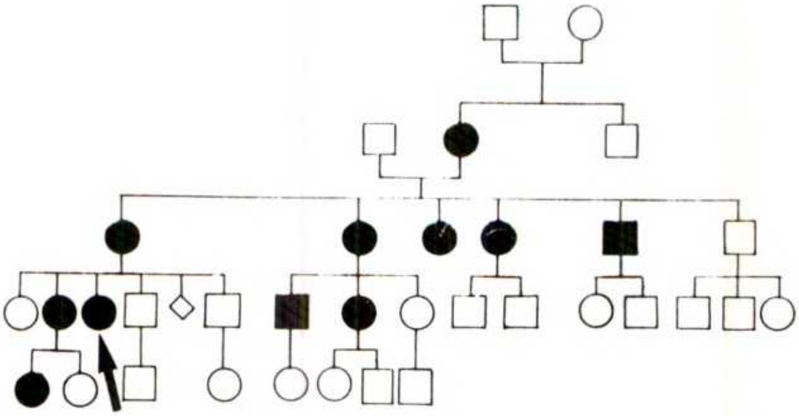

FIGURE 4. Family tree of patients with Dowling-Degos disease. The arrow indicates Case 1 in our report.
● Affected female; ■ Affected male.

DISCUSSION

DDD is a distinctive clinical entity which is commoner in women, since fifteen of the twenty patients hitherto described and nine of the eleven affected patients in the present family were women. In our family the autosomal dominant transmission suggested by Wilson-Jones & Grice (1978) seems obvious.

Diagnosis is not difficult once the disease has been considered. However, DDD may create problems of diagnosis both clinically and histopathologically even in patients who have skin disorders which are clearly unrelated to DDD. For example, in some of the patients with ashy dermatosis recently described by Person & Rogers (1981) there was a flexural and periorbital reticulate pigmentation, although the histopathology was clearly different from DDD. On the other hand, the patient described by Delacrétaz & Balsiger (1979) with multiple follicular hamartomas had completely different clinical features but histopathology identical to DDD.

It may be that DDD, as we know it today, is only one facet of a complex genodermatosis with variable expression in which many rare conditions, including perhaps Haber's syndrome (Wilson-Jones & Grice, 1978; Crovato & Rebora, 1982), may find a place.

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Dystrophic epidermolysis bullosa presenting with congenital localized absence of skin: report of four cases

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SUMMARY

Four cases of epidermolysis bullosa presenting with congenital absence of skin are described. These cases were shown to be dystrophic epidermolysis bullosa on clinical and ultrastructural findings. They demonstrate that congenital localized absence of skin (Bart's syndrome) may be the presenting sign of epidermolysis bullosa.

A syndrome was described by Bart (Bart *et al.*, 1966) with hereditary congenital localized absence of skin from the lower legs associated with blistering of the skin and mouth and nail abnormalities. The condition is inherited as an autosomal dominant trait. Later, Bart (1970) reported a patient with epidermolysis bullosa simplex who was born with congenital absence of skin, and Smith & Cram (1978) described a patient presenting with similar clinical findings but who appeared to be affected by dystrophic epidermolysis bullosa. This case was reported as 'A mechanobullous disease of the new born', and was claimed to be unique.

However, a similar clinical appearance was described by Herlitz (1934–35) in his original description of junctional epidermolysis bullosa, and another such patient whose diagnosis was confirmed by electron microscopy has been reported (Skoven & Drzewiecki, 1979).

We report four patients presenting with congenital localized absence of skin. Each patient later demonstrated typical features of recessive dystrophic epidermolysis bullosa. We conclude that congenital localized absence of skin may be a feature of any of the major variants of epidermolysis bullosa.

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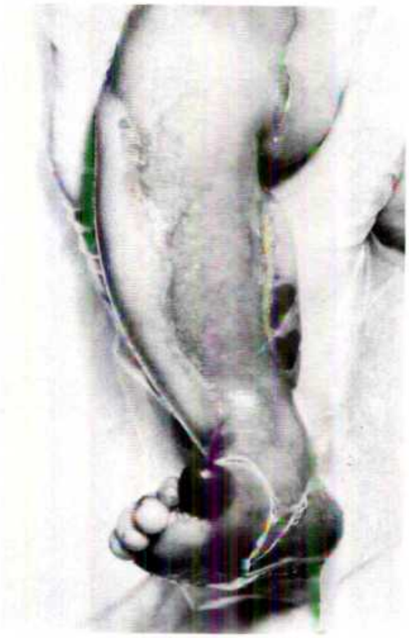

FIGURE 1. Case 1; appearance shortly after birth, showing large denuded area of leg and foot.

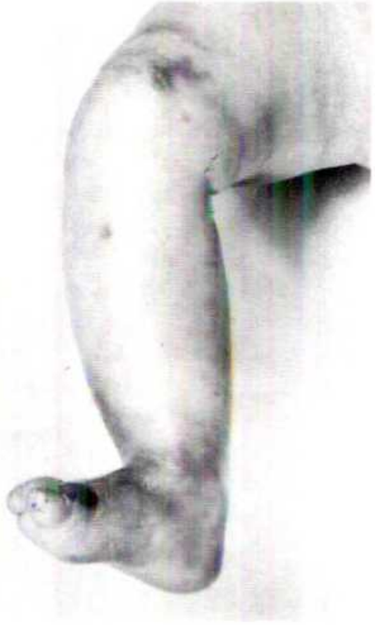

FIGURE 2. Case 1; the same leg as in Fig. 1, to show healing at 1 year.

CASE REPORTS

*Case 1, V.M.**

This 18-month-old girl presented at birth with large symmetrical areas on the lower legs and dorsa of the feet from which the skin was absent (Fig. 1). Blisters subsequently appeared at sites of trauma on the hands, trunk and in the mouth, and there were subungual haemorrhages. Prednisolone 10 mg on alternate days did not influence the development of blisters.

The denuded areas healed with little scarring (Fig. 2). She has continued to develop blisters which heal with scarring and milia formation and has lost nails.

There is no family history of blistering or congenital absence of skin. Her sister is normal.

Case 2, I.H.

This boy, aged 9 months, presented at birth with large eroded areas on the dorsa of both feet (Fig. 3). Shortly after birth he developed blisters on the elbows and hands and in the mouth, and had subungual haemorrhages.

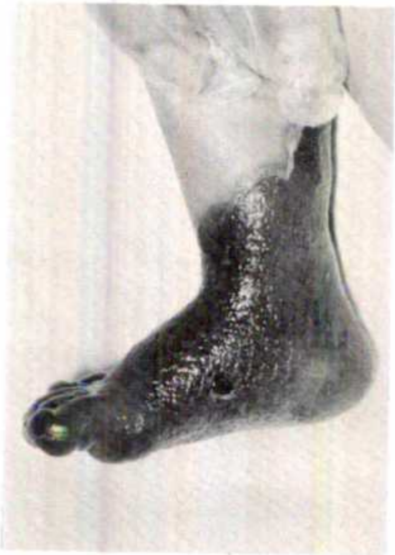

FIGURE 3. Case 2; denuded area on the lower leg shortly after birth.

Prednisone 10 mg daily prevented blistering, and a reduction in dose to 8 mg resulted in a relapse.

The eroded areas have healed. The blisters continued to form and heal with scarring, including fusion of the toes and milia formation. He has lost nails.

There is no family history of congenital absence of skin or blistering. There is an atopic sibling.

Case 3, V.H.

This 3-year-old boy was born with large areas from which the epidermis was absent on the dorsal and plantar surfaces of the feet and lower portions of the legs (Fig. 4), and with sloughing

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*Shown at Meetings of St John's Hospital Dermatological Society, June 1980 and of the British Association of Dermatologists, July 1981.

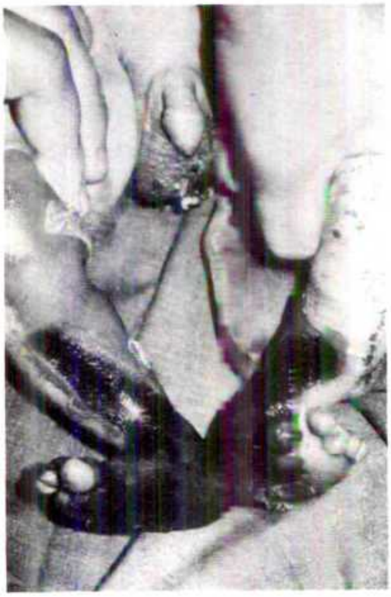

FIGURE 4. Case 3; large areas of legs and feet lacking the epidermis.

of the buccal mucosa. The next day ulceration of the mouth and lips became more severe, and blisters formed on the face, hands and trunk. He was treated with high dose systemic steroids (Dexamethasone 20 mg daily) without improvement, and it was discontinued after 7 weeks.

The eroded areas have healed. However, the subsequent clinical progress has been poor. He has had severe recurrent blistering resulting in milia formation, loss of nails, fusion of digits and limb contractures. He has marked stunting of growth.

There is no family history of blistering.

Case 4, A.P.

This 9-month-old girl presented at birth with denuded areas on the lower legs and dorsum of both feet. The denuded areas have healed, but she has had severe oral ulceration and severe blistering and scarring of the hands and feet, despite treatment with high doses of systemic steroids and phenytoin. She has failed to grow.

METHODS

In all cases, biopsies were obtained from the edges of blisters or perilesional skin, or from clinically normal skin. In one patient a biopsy was taken from normal skin that had been gently rubbed. The specimens were cut into small fragments and processed for light and electron microscopy. Primary fixation was carried out with 3–5% glutaraldehyde in cacodylate buffer with or without the addition of 2% formaldehyde, and post-fixation was performed with osmium tetroxide in s-collidine or phosphate buffer. After being dehydrated in ethanol, the tissue was embedded in Epon. One micrometer thick sections were stained with toluidine blue or Giemsa reagent for light microscopy, and ultrathin sections were stained with uranyl acetate and lead citrate for ultrastructural examination using JEOL 100CX electron microscopy.

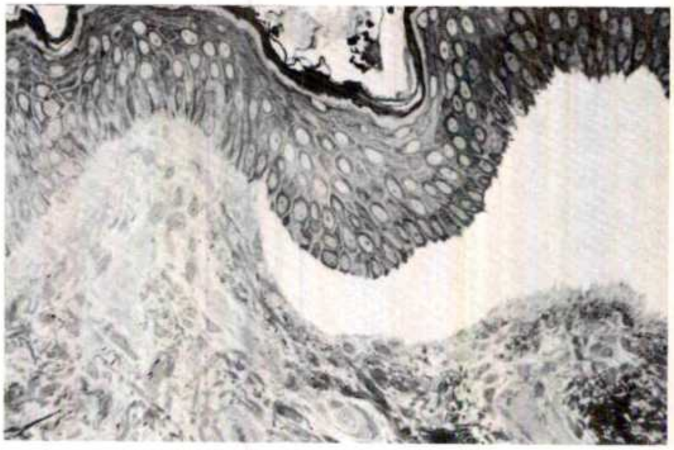

FIGURE 5. Case 1; photomicrograph of 1 μm thick Epon section showing clean junctional separation of intact epidermis from dermis (Giemsa stain, × 260).

RESULTS

Light microscopy

In sections from at least one biopsy sample from each case, there was a distinct cleavage in the dermo-epidermal junction (Fig. 5). The epidermis remained largely intact and was clearly separated from the underlying dermis, which also appeared generally normal.

Electron microscopy

The chief finding common to all four cases was that the level of dermo-epidermal separation was

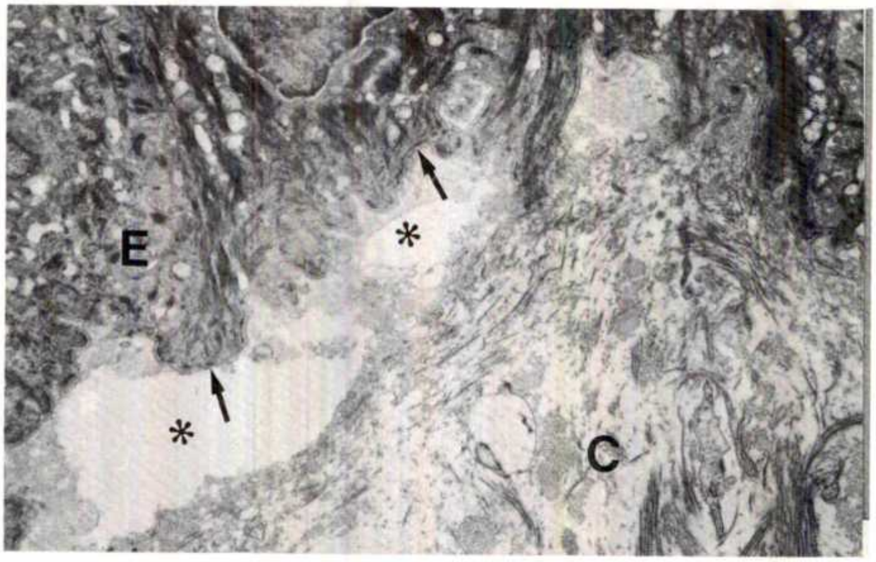

FIGURE 6. Case 3; electron micrograph showing early stage of dermo-epidermal separation caused by biopsy trauma in clinically normal skin. Epidermis (E) overlies dermal collagen (C) which is vacuolated (asterisks) immediately below the basal lamina (arrows) (× 5600).

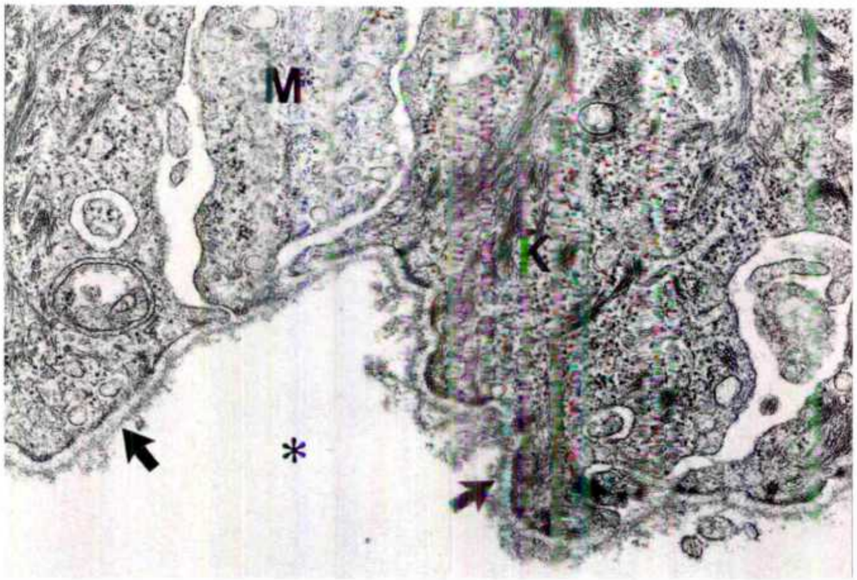

FIGURE 7. Case 4; electron micrograph representing later stage of dermo-epidermal separation. Structurally normal basal epidermal keratinocyte (K) and melanocyte (M) with underlying basal lamina (arrows) form roof of the cleft (asterisk) (× 20,800).

immediately below the basal lamina of the dermo-epidermal junction. In the early stage of separation, as noted in a sample of clinically normal skin, in which biopsy trauma was presumably the cause of the lesion, superficial dermal collagen appeared lysed (Fig. 6). In other samples where there was evidence of blistering clinically, the epidermis, with the basal lamina clinging to its under-surface, lifted cleanly from the underlying dermis (Fig. 7). In perilesional skin that was clinically normal, and where dermo-epidermal separation had not occurred, anchoring fibrils appeared wispy and poorly developed (Fig. 8). Other components of the

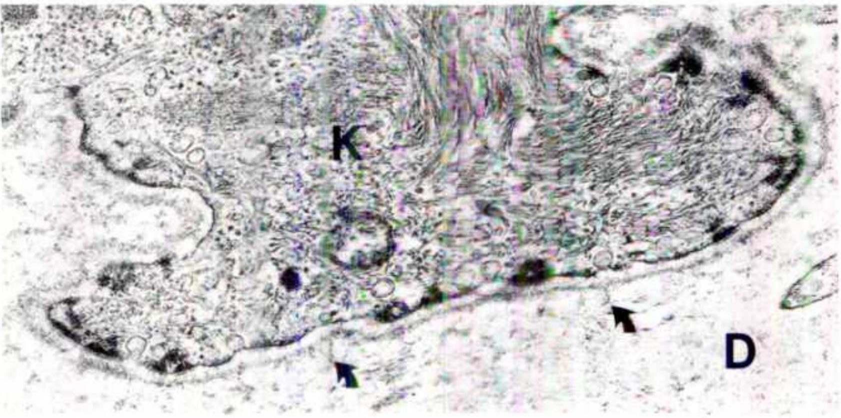

FIGURE 8. Case 4; electron micrograph of perilesional skin. Part of a keratinocyte (K) is united with the dermis (D). Anchoring fibrils (arrows) on the dermal side of the intact basal lamina appear poorly developed and possibly reduced in number (× 27,040)

dermo-epidermal junction, including hemi-desmosomes, were normal in ultrastructure. The observations relating to perilesional skin applied to each of the four patients.

DISCUSSION

The initial clinical presentation of these four patients was very similar to those cases in Bart's original description (Bart *et al.*, 1966). However, they differed in the absence of a family history and in their subsequent course.

The clinical diagnosis made in all our cases was recessive dystrophic epidermolysis bullosa. The grounds for this diagnosis were the absence of affected parents, the severe and widespread repeated blistering with the sequelae of scarring and milia formation and the involvement of mucous membranes and nails. The ultrastructural findings of dermo-epidermal separation below the basal lamina confirm that these cases are the dystrophic variant of epidermolysis bullosa. The absence of normal anchoring fibrils in peribullous skin are also suggestive of the diagnosis. We did not biopsy any of the lesions with the congenitally absent skin and are, therefore, unable to say what thickness of skin was deficient at birth. Bart (1966) originally felt that although congenital localized absence of skin was a recognized manifestation of junctional epidermolysis bullosa (Herlitz, 1934–35) his cases represented a distinct entity. He later suggested that they might be a form of epidermolysis bullosa (Bart, 1970). Our four cases confirm that congenital localized absence of skin may be a manifestation of epidermolysis bullosa and prove that it can occur in recessive dystrophic epidermolysis bullosa. The case of Skoven and Drzewiecki (1979) demonstrated that it occurs in junctional epidermolysis bullosa. It seems likely that epidermolysis bullosa simplex may also present in this fashion, as suggested by the photomicrography of the skin of a child with epidermolysis bullosa reported by Bart (1970).

Congenital localized absence of skin should be regarded as a manifestation of epidermolysis bullosa, and not as a separate specific entity or syndrome. The type of epidermolysis bullosa and subsequent course cannot be predicted from this presentation.

ACKNOWLEDGMENTS

We would like to thank Dr J. Kirby and Dr P. Betts for permission to report their patients. The electron microscopy on case 2 was performed by Dr Brian Lake.

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The Michelson Lecture

Delusions of parasitosis*

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Dr Michelson (Fig. 1) impressed his personality on, and was responsible for developing, the dermatological life of the University of Minnesota, while at the same time becoming a national and international figure. He did this not by self-advertisement, but quietly, as a disciplined man of few words but many thoughts, winnowed by a discerning mind; friendly to meet, but always commanding respect; not a man to cross lightly. Dr Lynch felt Dr Michelson was regarded highly because of his integrity and self-restraint (Lynch, 1972). Dr Grant Peterkin admired his conversation and erudition (personal communication). Dr Milton Orkin said he was a superb clinician and teacher (personal communication). Above all his partner, Dr Sheldon Mandel, held him in high esteem and affection (Mandel, 1976). He came of German stock, and his life blended to a marvellous degree the virtues of the New and Old Worlds. Contrary to the usual practice at that time he trained in the United States, and visited Europe secondarily, so that the formative influences of his early dermatological education were exclusively American. One of his many interests was psychosomatic disease (Michelson, 1945).

He is not an easy man for me to live up to: nor is this an easy subject. In fact *Delusions of Parasitosis* presents the most difficult problem of management in dermatology. It is also rare; so I decided I needed help, and wrote to 374 people, mostly members of the *British Association of Dermatologists*, asking for their experiences. May I say how grateful I am for their help, and that I hope my desire to record them faithfully has been successful.

My letter took the form of a general cry for help, asking specifically about the use of pimozide, the age and sex incidence, whether the delusion was really an isolated phenomenon, and did it ever resolve spontaneously? I also asked about outbreaks of imagined parasites in factories or institutions. I had considered sending a computer-type form with, as one correspondent punningly put it, little boxes to put ticks in, but rejected this because I felt too ignorant of the subject in general, and wished to stimulate the memory and imagination of my correspondents rather than to batter them with my preconceived ideas.

Two hundred and sixteen replied; twenty-three said they had never seen the problem. Most of these were young, but one, experienced and retired, stated he had always regarded it as 'no more than a minor derangement of rather impressionable people.' This remark was the first indication that such patients are not distributed equally among dermatologists.

The information obtained about 282 patients forms the basis of this retrospective study.

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*The Michelson Memorial Lecture, given to the Minnesota Dermatological Society in the University of Minnesota, Minneapolis, on 14 May 1982.

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FIGURE 1. Dr Henry E.Michelson, M.D. 1888–1972. Courtesy of Professor R.W.Goltz, Minneapolis. U.S.A.

Age and sex

There was a female to male ratio of two to one in the whole series, (1:1 under the age of 50, and 3:1 over 50). The youngest was a man of 19 and the eldest was a woman of 90.

Incidence

It is rare. The apparent decline noted by some dermatologists may be related to a particular interest being taken in it by others, who collect proportionately more patients. The largest number reported by one individual was thirty. Incidences are quoted from once in a lifetime to three times in the last year. It was estimated to be seven times rarer than dermatitis artefacta.

Not everyone wants to see them: 'I have only had the misfortune to handle three or four patients with parasitophobia' and 'I treat all mine with benzyl benzoate, and they never come back,' were two comments.

What kind of people are they?

'I was interested and surprised to find them quite intelligent,' wrote one dermatologist. Twenty-five patients were in professions, while seven were in manual occupations. The former group included six doctors (three in psychiatry). Many had strange personalities, and some were recluses. Social isolation often existed, and could be extreme. Many felt guilty, and some were melancholy. Two young men and one young woman were drug addicts.

Aggressive and paranoid suspicious traits stood out, because these impinge most upon the dermatologist's comfort and self-esteem. The grossest example was a mentally disturbed lady who, on lithium therapy, developed delusions of parasitosis, and then sexual delusions directed towards her dermatologist. She rang him up at all hours of day or night, broke into his lectures, seizing him and demanding to be kissed, broke into his parents' bedroom in the night and castigated them, and incited her alcoholic husband to cite the dermatologist as co-respondent in a divorce case. The dermatologist found her one day in his garden, brushing her hair over the dog, trying to infest it with her 'parasites'; he used to receive small packages of cockroaches through the mail, and find others attached to his car.

This story was exceptional, but scorn at the dermatologist's alleged incompetence was usual. For example, a lady had been describing the insects that crawled out of her skin, and then crawled back again. She had been disabused of her ideas, and on the way out of the consultation, her little husband peered round his bulky spouse, and said to the dermatologist, 'You miserable bastard.'

Some patients remain placid, in spite of their delusions. I suspect that paranoia or resignation are the extremes of a whole gamut of possible reactions to a world that refuses to take the 'parasites' seriously.

The onset

In six patients the delusion began with a real infestation, and in nine the start was blamed on an alleged infestation (from crab lice, cat parasites, midge bites, parasites disseminated by a travelling circus, or finding a mouse in the bed). Racial prejudice was evident in three: a white sea captain in a West African port, who blamed 'the locals'; a Scottish lorry driver, who blamed a dirty English toilet; and the alleged contraction of a West Indian parasite from an English swimming bath. Guilt over sexual indiscretions was evident in five patients, including a teacher of the classical guitar, who found himself in bed with his pupil's mother. Specific emotional stresses were blamed in five patients. One started with a stroke, another with hepatitis.

Skin lesions

Skin lesions were often absent (though some claimed they had them). There was a variety of traumatic lesions, varying in severity from tiny scratches to gouged out pits and ulcers. The excoriations might be generalized or localized, for example to the ear. Although widespread excoriations look fairly symmetrical as a rule, there is a degree of asymmetry imposed by the greater capabilities of the patient's master hand, which shows up well in the shoulder-blade region. One had prurigo nodularis, and one had razor slashes on the chest, inflicted because he found the presence of the 'parasites' intolerable, and had to let them out. A patient suffering from what she called 'coral mite dermatitis', had a skin stained by the dyes in which she habitually bathed. Two had incomplete alopecia, due to hair pulling, one shaved the scalp and body hair regularly, and two had onychotillomania.

The 'parasites'

Most patients provided specimens of their 'parasites' (Fig. 2). The dermatologist ought always to examine these carefully because on six occasions the parasites were real. 'I was handed a matchbox by a young man,' said one dermatologist, 'who told me he had been under a psychiatrist. He insisted that I looked at the contents of the match box, which I did with disdain and reluctance. He was suffering from pediculosis pubis.' A lady, who had been standing under a tree in California, selling fruit, complained that her scalp was full of flies. It was.

The specimens sometimes contained living things, such as thrips, spiders, flies, crane flies,

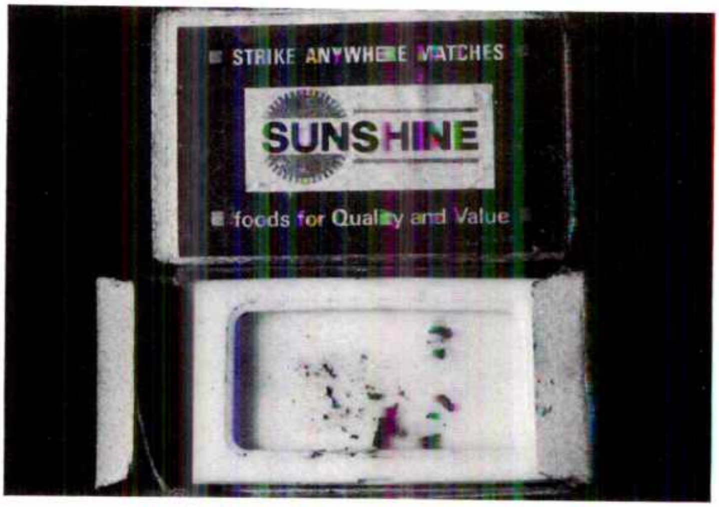


FIGURE 2. Specimen of 'the parasites' brought by a patient.

ants and cockroaches, but usually consisted of bits of skin and amorphous debris (Fig. 3). There were also bits of nail, human hair, animal hair rolled into a ball, beard hairs with the bulb attached (of which the patient had made a microscopic preparation, using his son's microscope set), sand, coal, fibre, cheese, bacon rind or bits of gold wire. Some specimens were bulky and coarse, others so sparse that it was difficult to find anything.

In my own experience, a piece of limp, dirty, much worn paper has been the usual container for the 'parasites', but most popular of all was a match box, while particularly fine plastic bags came from a psychiatrist. Sometimes the 'parasites' were said to move or jump, and this was demonstrated by a patient knocking on the arm of an 'infested' article of furniture, when the debris present naturally jumped up and down. One specimen was thirty colour photographs of skin, alleged to be the patient's own, in order to substantiate that he really had had an eruption.

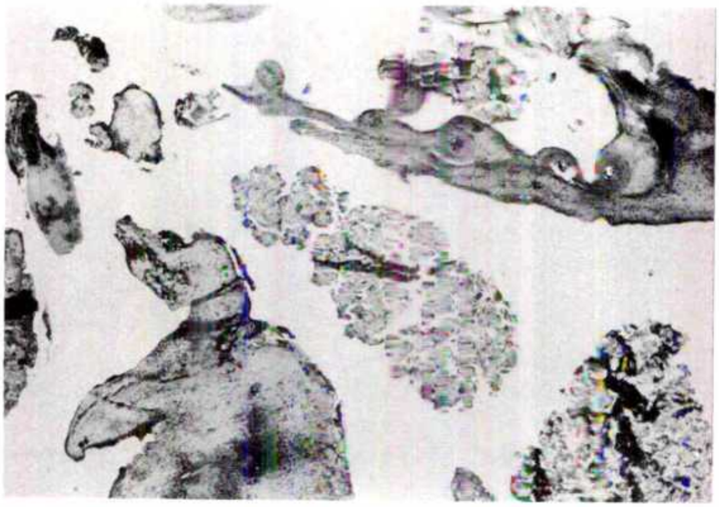


FIGURE 3. Histopathology of the specimen 'parasites' showing recognizable pieces of skin. Courtesy of Dr J.Savin, Edinburgh.

Stories about the 'parasites' vary from time to time, and it is possible to harbour several kinds at once. Thus, one lady described four types (black insects, lice, ticks and white fly) and she enlarged on their appearance, activities, and the places where they were to be found (Fig 4).

The 'parasites' could be found in any part of the skin, including the scalp and nails, appearing at or disappearing into any of the natural orifices, or could be described as present, but invisible: 'you can see them, but you cannot catch them,' as one Irishman put it. One of the 'parasites', was a worm, living in the patient's bottom, 'which doth come out at night, doctor, and he doth spit', because of which it was necessary for him to plug the hole with the *Daily Mirror*.

The descriptions are convincing only if accepted at their face value. A parasitology technician produced a circumstantial but imaginary life cycle of 'the parasites', and another patient, who was a draughtsman, drew 'the life cycle of the skin disease' and what he called 'the growth of the infective body', which shows a burrow penetrating the epidermis and running horizontally in the dermis (Fig. 5)—but neither drawing would satisfy any Board-certified dermatologist. Awkward questions, such as 'Why are there no parasites today?' are countered by ascribing to the parasites purely nocturnal habits, or, 'Why doesn't the treatment get rid of them?' by answering that the adults are killed, but the eggs hatch, and the young ones take over. The 'parasites' may go beneath the skin to escape destruction. Conversely, a hot bath may be a good way to make them surface, and in one patient the bathroom became the particular scene of attempts to dig them out. The pertinacity with which patients will cling to belief in their 'parasites' was illustrated by a physicist, who, when shown under the microscope that there was no living matter, said, 'Ah, but you should look under the *electron* microscope.'

Involvement of local authorities

The patients' stories can be convincing enough to persuade Local Authorities to have the house disinfested, or to replace furniture, which 'had to be destroyed' because of the 'infestation'. One 84-year-old lady had not only procured fourteen new mattresses in this way, but, for good measure, had written to the Minister of Health to complain that the dermatologist had raped her in the out-patient department.

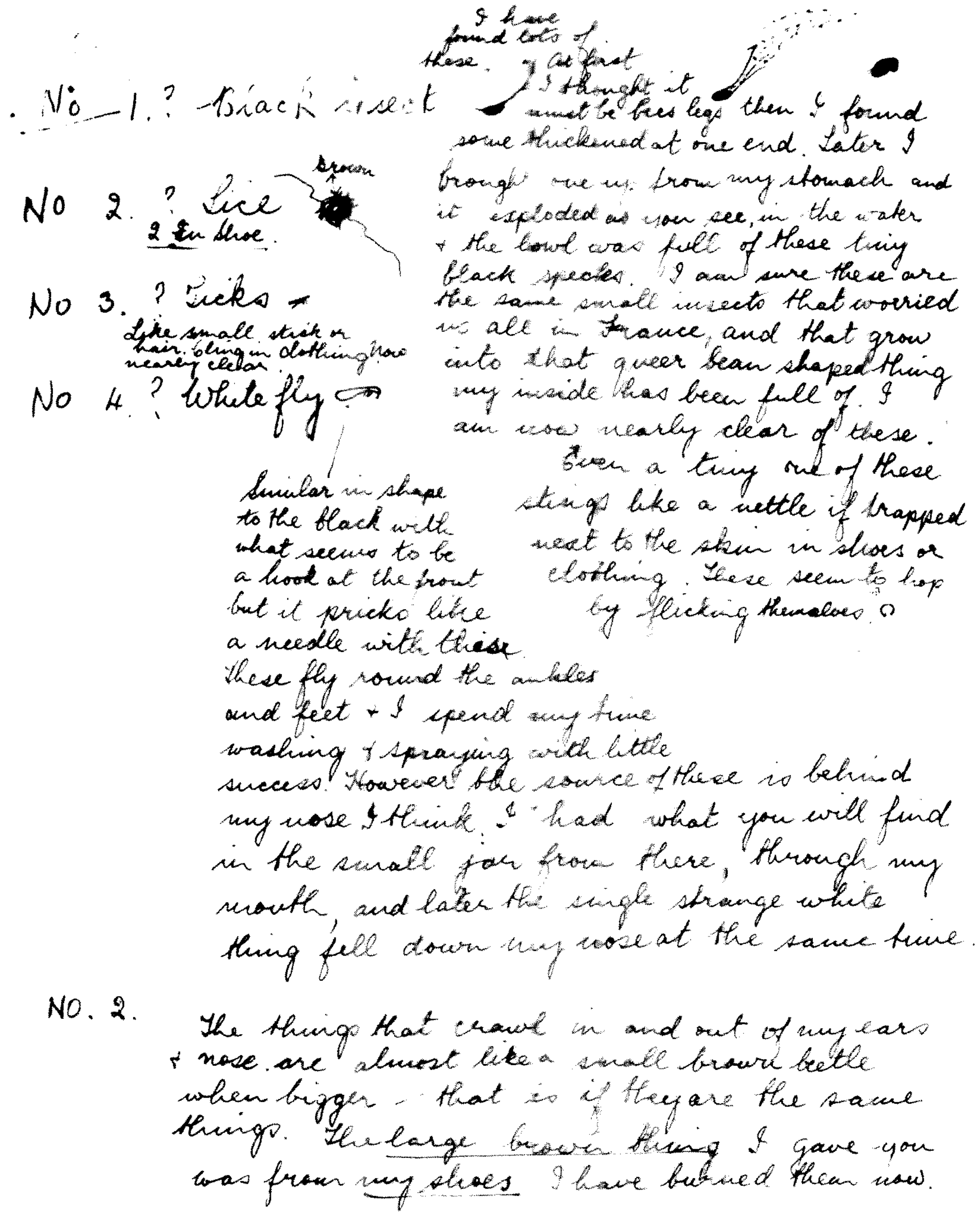
Involvement of entomologists and pest control firms

Pest Control Firms have learnt about these patients from bitter experience. Mr Edwards, an entomologist with Rentokil Ltd., received thirty-four specimens in his office in the period 1961–1978. Rentokil's policy is to refuse to treat the house unless definite parasites can be found, and to suggest that the client may have developed an 'allergy' to something, and advise him to see his general practitioner.

Mr Smith, an entomologist at the British Museum (Natural History) gets two or three enquiries every year. He has noticed that, though the individuals are unwilling to discuss anything except the 'infestation', there has usually been some emotional stress, which, in the elderly, may have been just loneliness. He noted that an anthropologist with a real occupational infestation produced no specimens of parasites for inspection.

Purification rituals and fear of transmitting infection

Patients wash compulsively, fear they will contaminate others, and destroy their 'infected' clothing. One had disinfectant brought to her in hospital so that she could 'wash herself properly', two had destroyed suites of furniture, and another had spent some thousands of pounds in renovation and painting. One had moved house three times in 3 years, and a mother and daughter eleven times in 4 years. A man, who believed he contaminated the seat of every



No 1. ? Black insect

No 2. ? Lice brown
2 in shoe.

No 3. ? Ticks
Like small stick or hair. Cling in clothing. Now nearly clear

No 4. ? White fly

I have found lots of these. At first I thought it must be bees legs then I found some thickened at one end. Later I brought one up from my stomach and it exploded as you see, in the water & the bowl was full of these tiny black specks. I am sure these are the same small insects that worried us all in France, and that grow into that queer bean shaped thing my inside has been full of. I am now nearly clear of these.

Even a tiny one of these stings like a nettle if trapped next to the skin in shoes or clothing. These seem to hop by flicking themselves.

Similar in shape to the black with what seems to be a hook at the front but it pricks like a needle with these. These fly round the ankles and feet & I spend my time washing & spraying with little success. However the source of these is behind my nose I think. I had what you will find in the small jar from there, through my mouth, and later the single strange white thing fell down my nose at the same time.

NO. 2. The things that crawl in and out of my ears & nose are almost like a small brown beetle when bigger – that is if they are the same things. The large brown thing I gave you was from my shoes I have burned them now.

FIGURE 4. Part of a letter from a patient describing the various kinds of parasite that infest her. Courtesy of Dr G.W.Senter, Birmingham.

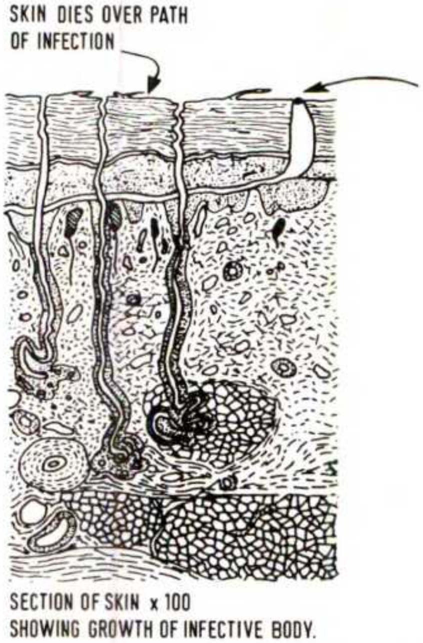

SKIN DIES OVER PATH OF INFECTION
SECTION OF SKIN x 100
SHOWING GROWTH OF INFECTIVE BODY.

FIGURE 5. Diagram drawn by a patient to explain the details of his infestation. Courtesy of Dr G.M.Levene, London.

lavatory he sat on, purified his own with a blow-lamp, to such effect that it eventually fell to pieces. This patient took his dermatologist to look at the waiting room. Many patients were rubbing their faces, or scratching themselves, and this, the patient insisted, was because he had passed on the contagion to them. He also produced a neighbour to testify that the whole street had been contaminated by him. One patient had stayed off work for months for fear of infecting his colleagues, while another chose to be self-employed, as this enabled him to control his contact with other people more easily.

Shared delusions

It is well known that delusions of infestation are 'infectious', the phenomenon being referred to as *folie à deux*, where two people are involved, and, when there are more, counting upwards in French as necessary. I prefer the terminology 'shared delusions', which does not specify any particular number of those participating.

Shared delusions were encountered in twenty-seven groups of patients, twenty-four pairs and three trios. The commonest pair was husband and wife (14), followed by mother and daughter (4), and mother and son (2). Father and son, sisters, woman and female companion, and divorced man and female lover were each encountered once.

Evidence of mental disease

These patients are reluctant to visit psychiatrists, and usually refuse to go altogether; but five had been seen on a routine basis by a psychiatrist, who worked in close collaboration with a skin department. He concluded that two were pre-senile, one depressed, a lonely woman had an obsessional neurosis, and the fifth was an emotionally unstable dullard.

Depression was diagnosed in twelve patients, schizophrenia in eight, while three suffered from unspecified psychoses. With the exception of these, my impression is that although many are eccentric, their eccentricity seldom crosses the ill-defined border of madness, except as regards their 'parasites'.

Evidence of physical disease

Two patients were noted to be atherosclerotic, and the delusion in one started after a stroke. Severe anaemia was mentioned five times, and in one instance its correction resulted in disappearance of the delusion. One patient was vitamin B_{12} deficient, but correction of this did not help. One had pellagra that responded to treatment with disappearance of the delusion. Severe renal disease was mentioned four times, one patient being on dialysis. The delusion in one coincided with an attack of broncho-pneumonia, and disappeared on recovery. Other coincident diseases included diabetes mellitus, multiple sclerosis and hepatitis.

Course and prognosis

This topic highlights a difficulty. How does one define delusions of parasitosis? If it is held that the delusion should be virtually permanent, the patient's conviction being immune to argument, the prognosis is naturally bad, if not hopeless. I suggested in my letter of enquiry that recovery might be possible by asking whether any remissions had been seen. I realize that in doing so, I have adopted the dictionary definition of a delusion as 'anything that deceives the mind with a false impression', rather than the technical one, which demands that the false impression be immutable.

There were thirteen reports of patients who had remitted. Three were of special interest: (1) A brewery worker had complained of crab lice for 3 months. There were no lice to be seen, and the specimen produced contained no parasites, but he flatly refused to believe this. He spent his time digging the 'lice' out of his skin, but said his efforts were frustrated because though he caught the adults, he couldn't keep pace with the eggs hatching. The dermatologist spent a long time talking to him, and impressed on him that malathion would kill the eggs as well as the adults. He failed to return for follow-up, but the general practitioner reported later that the itch had disappeared within 10 min of the application of malathion, and had not returned. This was clearly a cure by suggestion. (2) A District Nurse, in her forties, had been divorced recently, and was having problems with her teenage children. When she was told there were no parasites in the jam jar she had brought, she burst into tears, and unburdened herself of her difficulties. She made a good recovery from depression with treatment, and her delusion disappeared. She then confessed she had suffered a similar delusion previously under emotional stress. (3) A retired school teacher, aged 78, had been afflicted with the delusion for many years, and was estranged from her family because of it. She was examined carefully, and then confronted, in a confident manner, with the fact that she was deluded. Although she later admitted having been hurt by this directness, she nevertheless believed what she had been told, and was cured.

These examples refute the dictum, quoted by one dermatologist, that once the patient claims to see non-existent parasites, the case is hopeless, but I am not suggesting that remission is common. The delusion can certainly persist, and ruin the patient's life. A doctor of 53, deluded for 10 years or more, had become unfit for work, and had travelled the world seeking a cure, slovenly in his dress, and a mental wreck. Another man, in his thirties, with an apparently brilliant future, believed little insects with wiggling legs came down his nose. He was appointed to an important academic post, but had to give it up after a year or two because of his eccentric behaviour, and he never worked again.

Five of the patients had committed suicide.

As regards prognosis, it may be that if your delusion is a part of some other form of madness, your prognosis is that of that madness, and may be grave. If you are otherwise sane, there is hope for you, and the degree of hope has been increased by the advent of neuroleptic drugs, such as pimozide.

Differential diagnosis

It is important to make sure the patient is not infested. This is not always as easy as you might suppose. I have seen scabies overlooked in an elderly, intelligent lady, who had had the misfortune to have been labelled as delusions of parasitosis. One patient, who had been told she was deluded, proved to have head lice. A doctor's wife, who was complaining of parasites in her scalp, brought a specimen of them. 'Being a doctor's wife,' said the dermatologist, 'I thought I should look at it under the microscope; which I did, and found half a dozen dead fleas.'

Several correspondents pointed out that Asian and African patients tend to describe pruritus as 'things crawling in the skin', without giving it the particular significance that is implied by delusions of parasitosis, in which the 'parasites' are clearly visualized in the mind, and are seen to move about.

It is common knowledge that itching can be produced by the imagination. I can remember, when I started dermatology, itching all over every time I sat in the clinic. One of my correspondents described how, when he was a medical student, he showed his girl friend a flea under his new microscope. That night she itched and scratched in bed—unwitnessed, he added ruefully. No doubt this phenomenon is important in the pathogenesis of shared delusions.

There is an overlap with neurotic excoriations, prurigo nodularis, and artefactual disease. The gougings and excoriations of the deluded are indeed artefactual, but are conducted with the purpose of extracting the 'parasites, and are not concealed. Neurotic excoriations appear to have the function of relieving nervous tension, but a generalized and non-specific kind of tension, not one that has been organized into the specific shape of parasites. Two nurses had dermatitis artefacta, in addition to their delusions. The patient with prurigo nodularis was convinced that worms came out of his skin.

Management

According to one correspondent, the management of delusions of parasitosis can be divided into two epochs—B.P. and A.P—before pimozide and after pimozide. One dermatologist found that none of his patients did well on pimozide, but this experience was exceptional. Sixty-six patients had been treated, of whom forty-four responded, sixteen did not, and six were lost to follow-up. The response varied from total disappearance of the delusion, to some improvement, and included a state in which the delusion persisted, but the patient took no notice of it. A suicidal patient responded dramatically, within hours. The dose varied from 2 to 12 mg/day; beyond that, side-effects limited its use, as indeed they did sometimes within that range. Parkinsonism was mentioned in three patients (one necessitating admission to hospital), drowsiness in two, and weight gain in one. As a rule, relapse occurred on stopping, but control could be re-established on starting again. In one patient a remission lasted 2 years and two have shown no sign of relapse on stopping.

Ten dermatologists had found anti-depressive treatment of some benefit. One patient had been cured by an anti-depressant given under the guise of a new parasiticide. If the patient is depressed, anti-depressive treatment must be used in addition to pimozide.

One dermatologist asked who was to tell the patient that the parasites were imaginary. It may not be necessary to broach this thorny question, if the patient is satisfied that the dermatologist believes in the sensations. But statements that 'there are no parasites,' 'the parasites are imaginary,' or 'it's all in the mind,' are taken to mean you don't believe in the sensations either. You could say you are interested in the sensations, but you couldn't find any parasites today. If the patient would care to try these pills, which have helped other patients with a similar problem, you would like to see him again, and go into the matter further. Then you write a prescription for pimozide, 4 mg every morning.

These patients are said to do badly when referred to psychiatrists. Most dermatologists seem to refer as a matter of duty, or perhaps as a means of self-preservation, because they often add some such remark as 'does no good,' 'patient would not go,' or 'lost to follow-up.' Several admitted they lacked confidence with this type of patient. Most psychiatrists in Britain know less about this syndrome than dermatologists do. Referral to a psychiatrist is necessary if the mental abnormality extends beyond parasites.

We should not forget to pay attention to the skin, if this is dry, cracked, dermatitic, or secondarily infected. Emollients should be used, and the patient weaned from strong parasiticidal applications.

GROUP OUTBREAKS OF IMAGINED PARASITES

My interest in this subject was aroused by being called to a light engineering factory, because of what was believed to be an outbreak of some kind of parasite that was biting the workers and making them itch. The factory area had been sprayed twice already, without effect. When I arrived I was handed a bottle, said to contain 'the parasites,' with the added comment, 'you can see their wings.' The affected employees had little to see, and the little each had was ordinary in a different way (i.e. one had a scratch mark or two, another a few acne pimples, a patch of dermatitis, an ordinary mole, or nothing at all), producing the impression that there was no common cause. On going to the factory floor, I found all the cases were strung along a single production line, a distribution that was emphatically not what one would expect from some parasite loose in the factory, distributed, for example, in the ventilation system. I suspected this was a phenomenon of mass hysteria, and the idea was strengthened when the 'parasites' turned out to be metal filings. The outbreak settled with reassurance, and did not recur.

I included a request for information about factory or institutional outbreaks in my circular.

There were seven outbreaks of imagined parasites: they occurred in a hospital kitchen, a cosmetics factory, a zip fastener factory (confined to girls at a single table), the typing pool of a steelworks (attributed to low humidity), an operating theatre among technicians and cleaners, a hospital laundry (attributed to alarm and dependency generated by handling items of laundry marked with a new *Danger Hepatitis* label), and a clock and watch factory affecting the girls on a single assembly line.

'Phantom biters'

Mr Edwards, entomologist with Rentokil Ltd., refers to such outbreaks as 'phantom biters,' and believes there is usually a definite causative agent, though it may be difficult or impossible to identify.

A 2-year investigation of his into 'phantom biters,' done in co-operation with a large store, had shown that outbreaks follow periods of high temperature and high humidity, that they are commoner in the first half of the week, that older staff are less at risk than younger, and part-time than whole-time, that the legs and feet are the commonest site for lesions, and that biting insects cannot be found. Fragments of carpet fibre are believed to be responsible, but those who notice 'bites' when sitting or kneeling on the carpeted floor may have suffered static electricity burns. Textile fibres, fragments of glass fibre lampshades, or the coverings of wires on telephone switchboards, may be responsible in factories and offices. As regards control, spraying *during working hours*, even if just done with water, is sometimes effective.

These group outbreaks are usually brief, and do not involve the grimly held delusions seen in individuals suffering from delusions of parasitosis. This interesting subject has been neglected by dermatologists.

DISCUSSION

I have given full attention to the information entrusted to me by my colleagues, so that I have left little time for discussion. Perhaps that does not matter, because the findings of this survey are broadly consistent with what has been recorded in the literature (Perrin, 1896; Giacardy, 1923; Myerson, 1924; Wilhelmi, 1935; Ekbom, 1938; Wilson & Miller, 1946; Traver, 1951; Wilson, 1952; Ayres, 1953; Bers & Conrad, 1954; Dewhurst & Todd, 1956; McAndrews, Jung & Derbes, 1956; Tullett, 1965; Schrut & Waldron, 1963; Evans & Merskey, 1972; Bebbington, 1976; Gould & Gragg, 1976; *British Medical Journal*, 1977; Edwards, 1977), the only unfamiliar feature having been the production of photographs of the skin in support of the delusion. The rather haphazard way in which I have proceeded contrasts strongly with the orderly approach of Dr Annika Skott, a Swedish psychiatrist, who has written the definitive monograph on the subject (Skott, 1978), and has surveyed the literature thoroughly.

Perhaps the two most contentious issues are the aetiology of the delusion, and the validity of the term 'monosymptomatic hypochondriacal psychosis'. Psychiatrists see the content of a delusion as a personal matter (like a dream), giving some information about that person. As I see it, the delusion is the outcome of certain circumstances affecting a particular individual, and not some disease thrust arbitrarily upon him, commanding his obedience. Goltz & Derbes (1977) divided the causes of the syndrome into post-infestational, due to organic brain disease or to the effect of certain infections and drugs, occurring with psychoses such as schizophrenia and melancholia, connected with ageing, and idiopathic—all of which have been seen in this series. One could add psychoneurosis to this list, as Wilson (1952) did.

Having looked at many patients with this condition, my conclusion, like Dr Skott's is that many factors contribute. The younger the patients, the more clear-cut the aetiology usually is, whether it be schizophrenia, or drug addiction, or guilt, or being involved with a parent in a shared delusion. The older the patient, the more obscure the aetiological threads become. Too little attention has been paid to the patients' life situations, and also to the effects of age on their nervous systems and skins. Of course the patients don't help, because they blow up the accounts of their 'parasites' like some big, brightly coloured balloon, which they come into your presence waving to attract attention, obscuring other issues that from a medical point of view should have priority.

There are many possible reasons why an old person should feel strange goings-on in the skin. One of my correspondents, who was once a general practitioner, used to sit and talk to his elderly patients, and some of them described seeing strange little men under the bed at night. They would never have admitted this to most doctors, but they trusted him. Such misinformation, mediated by the senescent sensory system, could well be an important factor. Vitamin B_{12} deficiency (Pope, 1970), mulnutrition, pellagra (Aleshire, 1954), severe anaemia, multiple sclerosis, liver and renal disease could play a part. Arteriosclerosis, with or without diabetes mellitus, interferes with sensory function. One of the patients in this series began by having a stroke, and the famous lady described by Fauré, Rechfold & Ebtinger (1957), in whose ear lived *le grand capricorne*, was hypertensive, and strokes parallelled the step-like progression of her parasitosis.

We all know, if we pause to consider, that we often feel itchings or prickings in our skin, but these feelings are normally suppressed, so that they occupy us only momentarily and we don't really pay attention to them. It is likely that the skins of old people, which are often dry and flaky, itch and prickle even more; in fact this idea lies behind our belief in a senile type of pruritus.

But even so, why should sensations be transformed into a belief in parasites? As one of my correspondents points out 'you cannot have delusions about something you have never heard about, but you can have delusions about non-existent things that you have heard about.' The sensations need some framework of knowledge in which to expand, before the fairy tale of parasitosis can be told. Surely the fact that one patient had worked in parasitology, and another, who thought worms were coming out of his skin, worked on a maggot farm, is important? But why should other people, who have never had such occupational exposure, dream up the idea of parasites? A real infestation is a possible trigger. Hopkinson (1970) saw a schizophrenic patient develop delusions of infestation as an episode of her mental illness, after an attack of real scabies.

At one time lice may have been called 'the pearls of God', and the saintliness of Thomas à Beckett demonstrated by the great number of lice found on his body after his murder in Canterbury cathedral, so that his inner garment 'boiled over with them, like water simmering in a cauldron' (Busvine, 1976). But these days parasites evoke feelings of repulsion, horror, disgust and disgrace. In this series feelings of defilement and guilt following sexual indiscretions have been important in some patients. But we cannot really suppose that this mechanism explains the sudden increase in incidence that occurs from the 60s to the 70s. I believe one factor could be the fear of death. When we are young, life spreads in front of us towards an infinitely distant horizon, but as we age, the knowledge that we have to die comes to live with us. Men and women meet this universal threat in different ways, according to their various personalities and beliefs. But, for all thinking people, this is a time to look back at what has been, to relive past experiences, to stir vain regrets for what might have been, and sometimes to raise a spectre of guilt, and a foreboding of wrath to come. With such thoughts in the mind, could not a lonely, elderly person interpret what she feels as parasites feeding on her corrupt flesh, a prelude to death and judgment? Was it a coincidence that a retired school teacher, who had been helping to tidy a cemetery, should start to feel worms coming out of his skin? But I am aware that such apocalyptic thoughts are out of fashion, and it may be more simply that the old soul, beset by strange feelings in the skin, fears she is going mad, and so her mind invents a physical explanation—parasites. Hence her insistence on their reality, the detailed description of them, the actual specimens, the demonstration that they actually do move, and her refusal to go and see the doctor appropriate to mad people.

The doctrine of monosymptomatic hypochondriacal psychosis and the use of pimozide can be considered together, for the great point made by Munro (1978), who is a chief proponent of this syndrome, is that patients suffering from it can be expected to respond to pimozide. The theory of this doctrine is that the psychotic element is limited to one facet of the patient's life, whether it be the presence of imagined parasites in the skin, the allegedly abnormal shape of his face, or the foul smell that he believes exudes from him. Beyond this he or she is normal, or at any rate within the bounds of acceptable eccentricity. Certainly there is considerable support for this idea among some of the patients in this series, but it remains to be seen whether the concept of such an isolated delusion will stand up to the test of broadening psychiatric experience. That pimozide is helpful in some patients with delusions of parasitosis, there can be not doubt, both from the results of this series, and what has been reported in the literature by Munro (1978) and by Reilly, Jopling & Beard (1978), by Hamann & Avnstorp (1982), who conducted a convincing double-blind crossover trial, and by Hamann (1982) who documented a dramatic cure of onychotillomania. Pimozide is a great advance and also, perhaps, a pointer to some chemical mediator being involved.

Finally, I would like to hand on to you a discovery made by Miss Teresa Matthews, a medical secretary. She found, in *Aubrey's Brief Lives*, the biography of one James Harrington (1611–1677) (Fig. 6) who was imprisoned in the Tower of London in 1660, for having tried to change the Constitution. His life had been wracked by frustrations. A republican at heart, he had been personal attendant to Charles I, had liked him, and had been with him on the scaffold. On the other hand, the publication of his book *Oreana*, which set out his political beliefs, had

FIGURE 6. James Harrington 1611–1677. Courtesy of the National Portrait Gallery, London.

been delayed by Cromwell until 1656, and when it did appear, it caused a furore. His imprisonment, said Aubrey, brought on his madness, 'which was not of a raging kind, for he could talk rationally, and was excellent company; but he began to imagine that his sweat turned to flies, and sometimes to bees and other insects. He had a revolving summer house, lined with cloth, built in his garden, so that it could be turned towards the sun. The warmth would draw flies out of the cloth, and then he would cry: "Don't you see that these flies come from me?" It was the strangest madness that ever I found in anyone.'

ACKNOWLEDGMENTS

I am grateful to Professor R.W.Goltz, for having invited me to give this lecture. I am grateful also to all my correspondents, too numerous to mention by name, many of whom went to immense trouble to help me, and without whom this would have been but a lean tale. I am grateful especially to Dr I.B.Sneddon for putting his knowledge at my disposal so unselfishly, to Dr J.O'D.Alexander and Dr W.Fabisch for reading the typescript from the points of view of a dermatologist and psychiatrist respectively, to Mr R.Edwards and Mr K.G.V.Smith for entomological information and advice, and to Dr K.Hamann for information about pimozide and for letting me have a preview of his paper on onychotillomania.

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Correspondence

Generalized racemose livedo and neurological lesions

Sir, We were interested to read the report from Dr Thomas and his colleagues on livedo reticularis and neurological lesions (*British Journal of Dermatology* 1982, **106**, 711).

Our experience is that neurological disease is not infrequent in idiopathic livedo but the classification of livedo seems inadequate. Ehrmann (1907) distinguished livedo reticularis from livedo racemosa. Racemose livedo is characterized by a tendril-like bluish pattern reminiscent of forked lightning whereas livedo reticularis presents a fine net-like pattern. This separation seems to be important, since cerebro-vascular disturbances appear almost exclusively in generalized racemose livedo (GRL).

In a review of the literature we found details of thirty-three patients suffering from GRL of whom twenty-four (73%) had neurological lesions (Lubach & Stamm, 1981). In other forms of livedo, cerebro-vascular disturbances occurred very rarely.

In the last 4 years we have observed nineteen patients with GRL (fifteen women, four men) of whom fourteen (74%) had a neurological disorder. In twelve of these, cerebral infarction could be established and several patients had multiple cerebral infarctions. Encephalo-arteriographic examination showed constriction of the peripheral cerebral vessels. Scanning tomography enabled us to see diffuse atrophy of the cortex in addition to brain defects (Lubach & Stamm, 1982).

Skin biopsies from the livedo area showed endotheliosis of the arterioles in the deep dermis and occasionally thrombosis of the same vessels. Many patients showed no change in their vessels. In one patient we found endotheliosis of the same kind of arteriole in an iliac crest biopsy. No immunological or biochemical abnormalities were detected in our patients. We believe that GRL is a disease of systemic vessels similar to thromboangiitis obliterans. It mainly affects fairly young women and it attacks skin and cerebral vessels at a very early stage. It is possible that GRL has become more common in recent years and we estimate that there are about 1000–1500 patients with GRL in the Federal Republic of Germany.

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Squamous carcinoma induced by topical idoxuridine therapy?

Sir, We should like to report the following case. A 26-year-old white female dentist suffered from an especially aggressive, ulcerating lesion occurring centrally on the lower lip. The condition, which developed following sun exposure at high altitude, persisted for 2 weeks and was clinically diagnosed as herpes simplex. On recurrence approximately 2 months later Iduridin® ocular ointment (IDU) (1 g contains 2 mg idoxuridine) was applied to the lesion 3–4 times a day for 4 days, with reportedly good effect. However, a small tumour developed at the site of the lesion. This was excised and histology showed a squamous cell carcinoma (Fig. 1). Re-excision biopsy was devoid of tumour tissue. There is no recurrence after 6 years.

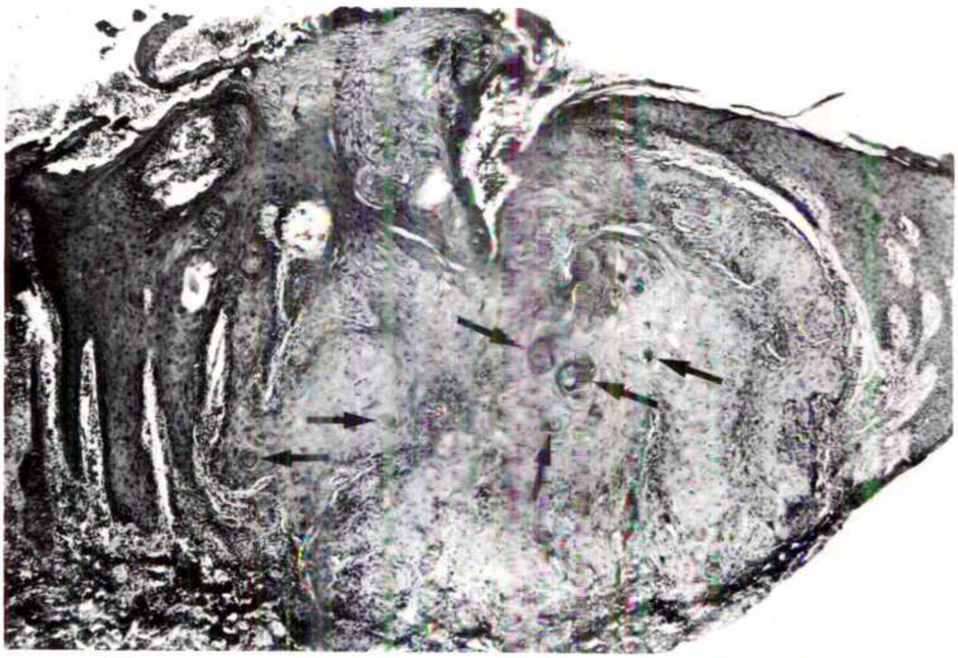

FIGURE 1. Microphotograph of vermilion border biopsy. The squamous epithelium shows an area of surface hyperparakeratinization and tumour proliferation to the deep resection margin. Arrows indicate keratin pearls (H & E, × 8).

A similar case was reported by Thompson & O'Neill (1976), who found premalignant changes after local application of IDU in dimethylsulphoxide (DMSO) on a clinically diagnosed penile herpes simplex lesion in a 20-year-old patient. Complete remission was seen after the treatment was discontinued.

Although extensively used in the therapy of DNA-virus infections (Juel-Jensen & McCallum, 1972) and clinically tested without observed adverse effects (Juel-Jensen & McCallum, 1974), an increased frequency of apparent allergic reactions and the occurrence of premalignant changes (Thomson & O'Neill, 1976) after local application of IDU in genital herpes simplex have prompted strong warnings against widespread dermatological use of the agent without further controlled studies (Løkken, 1975; Green & Staal, 1976).

Both IDU and DMSO have resulted in premalignant changes in cell cultures (Malinin, 1973; Malinin & Perry, 1967), and IDU has been described as potentially teratogenic, carcinogenic (Weinstein & Chang, 1973; Wright, 1973), and radiosensitizing (Djordjevic & Szybalski, 1960). DMSO is not incorporated in Iduridin® ointment and so a possible effect of this substance is excluded in our case. The possible carcinogenicity of herpes simplex virus is another aspect to be considered. At present, however, there is little basis to assume such a relationship in the oral region, where lesions are caused by herpes simplex virus type 1.

Conclusions as to the carcinogenicity of IDU cannot be drawn based on only two cases with so many factors involved, but we want to emphasize the extremely rare occurrence of premalignant and malignant changes in the regions under consideration in such young patients. A lip cancer in a 26-year-old female would be an extreme rarity in Norway.

We wish to repeat the warnings (Løkken, 1975; Green & Staal, 1976) and urge reluctance when considering IDU therapy in simple cases of herpes simplex.

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Hair loss following lithium therapy

SIR, I read with great interest the report by Dawber & Mortimer on hair loss during lithium treatment (*British Journal of Dermatology* 1982, **107**, 124) since I too had noticed this side-effect in psychiatric patients. I had found only two previous cases in the literature (Vacaflor, Lehmann & Ban, 1970; Vacaflor, 1975).

One hundred patients attending a lithium clinic were surveyed and followed-up for up to three years. Twelve of these patients complained of hair loss. Hypothyroidism was detected in three patients and two had regrowth of hair with thyroxine. The only male patient was identified as having loss of scalp hair because on reducing lithium he had some regrowth, and 1 year after stopping lithium he had regrown a full head of hair. A female aged 61 who had previously never noticed hair loss developed bald patches and took shelter in a wig. Two years after cessation of lithium her hair had regrown.

Three of the twelve patients complained of loss of body hair. In two this was marked, and one of these patients had pre-existing eczema. The third patient is of considerable interest since she had had alopecia areata since the age of 5 years. When started on lithium for manic-depressive psychosis at the age of 37 she developed alopecia totalis and within 2 years the condition had progressed to alopecia universalis, which seemed similar to the case mentioned by Vacaflor (1975). The patient had never had total loss of scalp hair before, nor any loss of body hair and she and her husband suspected this was due to lithium. Lithium was stopped and within months she developed regrowth of hair for the first time in several years. Admittedly this was restricted to vellus hairs on the nostrils and scalp. Some 3 years after cessation of treatment she had sparse regrowth of terminal scalp hair in a few isolated patches.

In conclusion it can be confirmed that hair loss may be noticed after only a few months' lithium treatment. There is no reason to suspect excessive dosage as the cause, as in only one of the twelve cases was the serum lithium level found to be above the therapeutic margin, and this was only for a short period. It can also be confirmed that withdrawal of lithium usually leads to a regrowth of hair.

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REFERENCES

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Epidermal nuclear immunofluorescence

SIR, Iwatsuki *et al.* (*British Journal of Dermatology* 1982, **107**, 653) reported that the speckled epidermal nuclear fluorescence seems to develop as an *in vitro* phenomenon. This was based on an *in vivo* study in which RNP antibodies were injected intradermally. The authors did not mention at what distance from the point of injection the biopsies were taken. They did not study the diffusion within the overlying epidermis of proteins injected under pressure intradermally. Therefore, they could not rule out the possibility that the binding of RNP antibody observed as early as 1 min after intradermal injection was due to an *in vivo* diffusion of the serum within the epidermis. A positive reaction was still present 48 h after intradermal injection of the RNP antibody. It is possible that a sufficient amount of RNP antibody was retained in the dermis. This is unlikely, but should be documented. Since the only point supporting the authors' theory is the fact that they observed a binding 1 min after injection, such controls would have been useful.

The methods used by Iwatsuki *et al.* do not represent an advance in this field, and do not add to the concept which has been well documented by the studies of Alarcon-Segovia *et al.* (1978) on mononuclear cells and confirmed by our group on keratinocytes *in vitro* (Galoppin & Saurat, 1981).

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ALARCON-SEGOVIA, D., RUIZ-ARGQELLES, A. & FISHBEIN, E. (1978) Antibody to ribonucleoprotein penetrates live human mononuclear cells through Fc receptors. *Nature*, **271**, 67.

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British Journal of Dermatology (1983) **108,** 505.

Book Reviews

Neurofibromatosis (Von Recklinghausen Disease), Genetics, Cell Biology and Biochemistry. Edited by V.M.RICCARDI and J.J.MULVIHILL (1981) Advances in Neurology **29,** New York: Raven Press. Pp. 304.

This volume comprises a collection of papers presented at a workshop on neurofibromatosis. The initial section will be the most interesting to dermatologists, comprising a review of the clinical and pathological aspects of the disease and its natural history, with special reference to its neoplastic associations. There follow several papers on the biology of the neural crest, which are in my view marred by the absence of any real discussion of the obvious question, whether neurofibromatosis is really a disorder of the neural crest or not, particularly since some clinical and pathological data suggest that it may not be. Much evidence points to the importance of combined genetic and humoral influences in neurofibromatosis; perhaps the most convincing clinical clue to this is the tendency for the disorder to be most severe in the offspring of affected mothers. The next section of the book is devoted to the various growth techniques that may be helpful in future research, monoclonal antibodies for example. The penultimate chapter contains an interesting discussion of some genetic aspects, particularly the possible differences between familial and non-familial cases. Finally, several authors join in a revealingly inconclusive debate on minimal diagnostic criteria for neurofibromatosis, which serves to highlight the many questions that still hang over this disease.

The intended audience for this volume presumably consists of neurologists and neuro-biologists primarily. Nevertheless, it contains a great deal of interest to dermatologists. It provides the most up-to-date account available of the biology, pathology and clinical behaviour of neurofibromatosis, and is an excellent reference source for those interested in almost any aspect of this intriguing disease. I doubt whether many dermatologists will go out and buy a personal copy, but this volume is a must for medical and dermatological libraries.

DAVID J. ATHERTON

Orale Photochemotherapie. By F. GSCHNAIT. (1982) German text. Vienna: Springer-Verlang. Pp. 184. Price Austrian S 620, $39.10.

This slim volume of some 180 pages is a valuable manual on PUVA therapy. The author was very early in the field, and the PUVA methods of treatment are now well known. The nature of UVA and UVB is discussed and the indications for treatment are defined. There are chapters on the use of PUVA in various forms of psoriasis, and the pros and cons of PUVA versus conventional therapy in various forms of the disease are fully dealt with. There are authorative accounts of PUVA therapy in polymorphic light dermatoses, mycosis fungoides, lichen planus, atopic eczema and vitiligo.

A most interesting chapter on side-effects of PUVA considers the association with tumours, cataracts and liver damage. The use of PUVA in combination with other treatments is discussed, particularly in association with retinoids.

The organisation of PUVA facilities within a hospital is also described. With every new treatment there are problems, and PUVA is no exception. Every clinician who gives PUVA or requests PUVA treatment for a patient will find this volume of great value in the everyday management of such patients. It is a timely, and most valuable guide, based on experience.

MAURICE GARRETTS

British Journal of Dermatology (1983) **108**, 506.

News and Notices

Honorary Fellowship of the Royal Society of Medicine

Dr Marion B.Sulzberger was recently made an Honorary Fellow of the Royal Society of Medicine (London). Dr Sulzberger is Professor Emeritus of Dermatology at New York University and at the University of California, San Francisco. He is the first foreign dermatologist to receive this distinction, which is limited to one hundred members who have distinguished themselves in medicine and related sciences.

Raymond Horton Smith Prize 1982

Dr Richard Rycroft has been awarded the Raymond Horton Smith Prize of the University of Cambridge for the best MD Thesis of 1982, entitled 'Soluble oil as a major cause of occupational dermatitis'.

British Society for Investigative Dermatology, Nottingham, October 1983

The Annual Meeting of the British Society for Investigative Dermatology (formerly known as the Investigative Group of the British Association of Dermatologists) will take place in the University of Nottingham on 30 September–1 October 1983. The programme will include sessions for both oral and poster communications. Abstracts should be submitted on the new official form and must be received by Dr R.A.J.Eady by 17 June 1983. For further information and abstract forms please write to: Dr R.A.J.Eady, Chairman, B.S.I.D., Institute of Dermatology, Homerton Grove, London E9 6BX.

Gold Medal for Allergy Research 1983

The Foundation for Allergy Research in Europe (F.A.R.E.) bestows a Gold Medal every 3 years for scientific work of practical clinical relevance in the field of allergy research. The next Gold Medal will be awarded during the XIIth Congress of the European Academy of Allergology and Clinical Immunology in Rome, September 25–30, 1983. This occasion will be combined with a guest lecture by the prize winner.

The topic chosen by the F.A.R.E. for the Gold Medal 1983 is 'Importance of Immunocomplexes in Clinical Allergy'. Participation is open to young scientists working in Europe, independent of their nationality or their type of work. Unpublished or recently published papers about the chosen topic may be submitted provided that they have not been submitted elsewhere for a prize or similar award.

Applications together with a *curriculum vitae* and list of publications should be in the hands of the secretary of the F.A.R.E., Prof. B.Wüthrich, Allergiestation, Dermatologische Universitätsklinik, Gloriastrasse 31, CH-8091 Zürich, Switzerland, before 1 May 1983. The council of the Foundation can choose the prize winner from among the candidates, but can also award the Gold Medal to a scientist for his/her scientific work in general if none of the papers presented are found worthy of the Gold Medal. Encouragement of young scientists has obvious priority.

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Pediatric Dermatology is a new international journal which is closely associated with the Society for Pediatric Dermatology and the International Society of Pediatric Dermatology. Its primary purpose is to publish original papers relating to clinical and laboratory investigations, case reports, review articles relevant to the speciality of pediatric dermatology, editorial comments and book reviews.

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Manuscripts should be sent to the Editor, Dr J.L.Burton, Dermatology Department, Bristol Royal Infirmary, Bristol BS2 8HW. They should be typewritten on one side of the paper only, with a wide margin, be double spaced, and bear the title of the paper, name and address of each author, together with the name of the hospital laboratory or institution where the work has been carried out. The name and full postal address of the author who will be responsible for reading the proofs should be given on the first page. A duplicate copy, complete with figures, tables and references, should be sent, since in many cases two referees are consulted. The author should keep a carbon copy of his manuscript. The Editorial Board reserves the right to make literary corrections.

Abbreviations and units. Full names with uncommon abbreviations must be given with the first mention: new abbreviations should be coined only for unwieldy names and should not be used at all unless the names occur frequently. In the title unusual abbreviations should be identified, in the introduction and discussion they should be used sparingly. Abbreviations of units should conform with those shown below.

gram(s)	g	minute(s)	min	molar	mol/l
kilogram(s)	kg	centimetre(s)	cm	millilitre(s)	ml
milligram(s) (10^{-3}g)	mg	second(s)	s	gravitational acceleration	***g***
microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm^3	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
hours(s)	h	milliequivalent	mmol		

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Package quantity: 250 ml polythene bottles with a 10 ml measuring cap.
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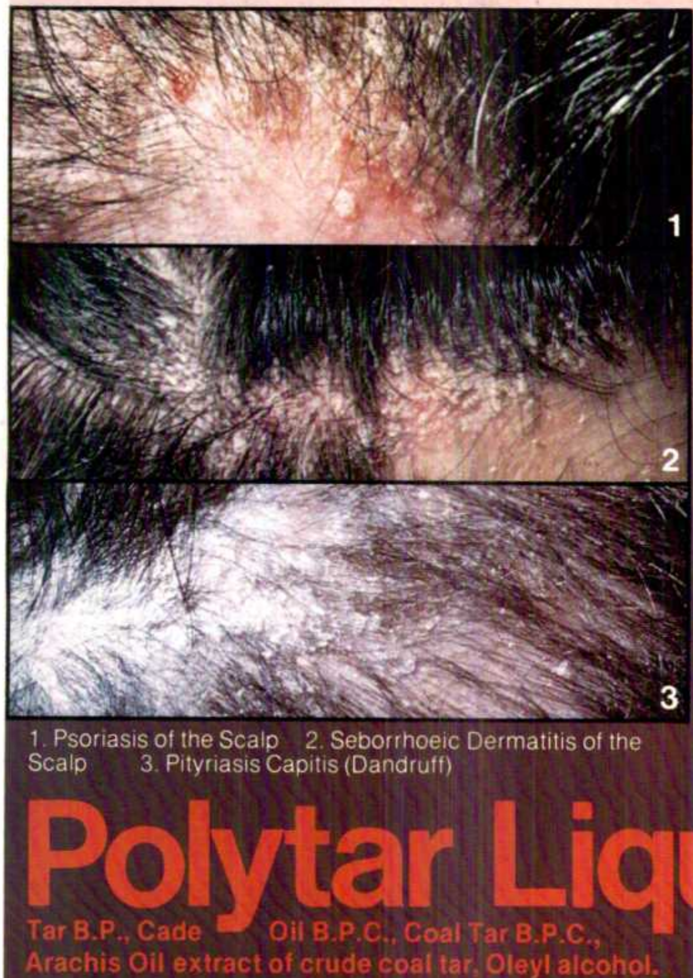

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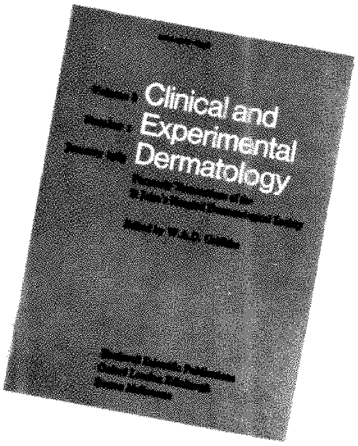

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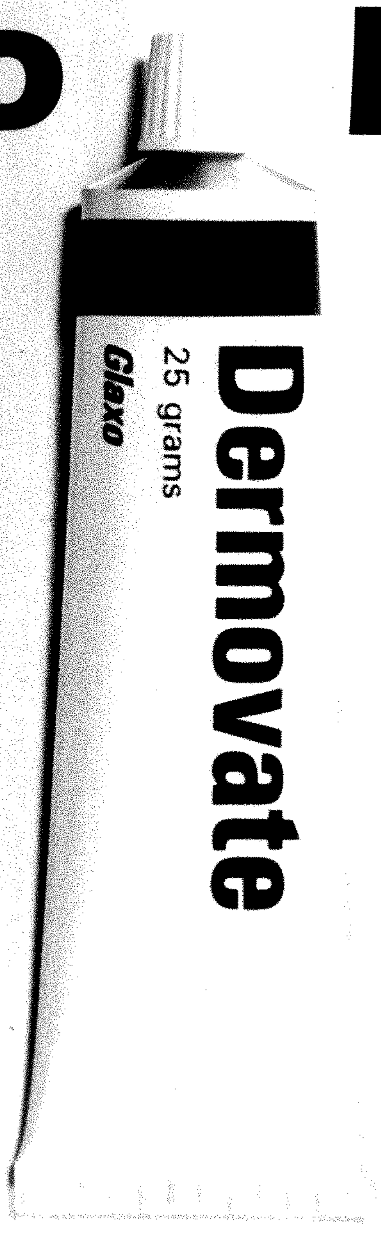

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Fry L. Update: Mar 1978 681-69

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1981. 236 pages, 113 illustrations. Paper, £7.50

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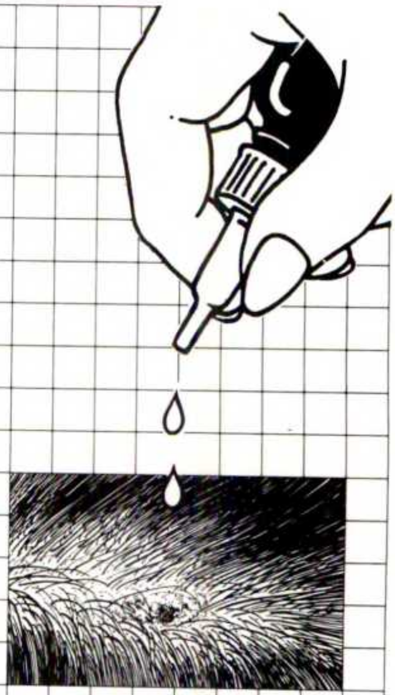

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Arthur Rook MA, MD, FRCP *Consultant Dermatologist, Addenbrookes Hospital, Cambridge*

and **Rodney Dawber** MA, MB, ChB, FRCP *Consultant Dermatologist, John Radcliffe Hospital, Oxford*

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1982. 567 pages, 197 illustrations. £35.00

Contents: The comparative physiology, embryology and physiology of the human hair; Hair follicle structure and keratinisation and the physical properties of hair; The hair in infancy and childhood; Hair patterns: baldness and hirsutism; Diffuse alopecia: endocrine metabolic and chemical influences on the follicular cycle; Hereditary and congenital alopecia and hypotrichosis; Defects of the hair shaft; Hypertrichosis; Traumatic alopecia; Alopecia areata; Cicatricial alopecia; Hair colour; Infections and infestations of the hair and scalp; Psychological factors and disorders of the hair; Hair cosmetics; Hair and scalp in systemic diseases; Diseases of the scalp and skin diseases involving the scalp; Naevi, tumours and cysts of the scalp; Investigation of hair and hair follicle diseases.

Blackwell Scientific Publications

Essentials of Industrial Dermatology

Edited by **W.A.D. Griffiths** MD, MRCP
Consultant Dermatologist, St. John's Hospital for Diseases of the Skin and Senior Lecturer, Institute of Dermatology, London

and **D.S. Wilkinson** FRCP
Consultant Dermatologist, Wycombe General Hospital

This compact handbook provides guidelines and sets out basic principles in the field of industrial dermatology for doctors working as full- or part-time factory doctors, who may have little previous dermatological training. It will also appeal to doctors working in public or community health, particularly in the developing countries where dermatological problems are becoming increasingly apparent. The text includes line drawings and tables for easy reference.

Contents: A national problem *W.A.D. Griffiths;* Industrial dermatitis today and its prevention *D. Burrows;* Constitutional skin diseases in industry *J. Cotterill;* Investigation of industrial dermatitis *C. Hindson;* Soluble oils *R. Rycroft;* Hand eczema in industry and the home *R. Church;* Chemical acne *D. Williamson;* Principles in dermatitis litigation *R. Bursell;* Conclusions and key points *D.S. Wilkinson & W.A.D. Griffiths.*

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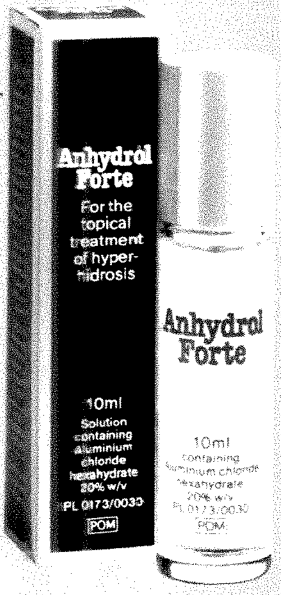
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British Journal of Dermatology (1983) **108**, 507–518.

Clinical and Laboratory Investigations

A comparison of the phototumorigenic potential of 8-MOP and 5-MOP in hairless albino mice exposed to solar simulated radiation

A.R.YOUNG, I.A.MAGNUS, A.C.DAVIES AND N.P.SMITH*

Institute of Dermatology, Department of Photobiology, Homerton Grove, London E9 6BX and * Department of Histopathology & Experimental Pathology, Lisle Street, London, WC2H 7BJ

Accepted for publication 2 August 1982

SUMMARY

Hairless albino mice have been treated with topically applied 8-methoxypsoralen and 5-methoxypsoralen at two concentrations and exposed to solar simulated radiation. Both these compounds significantly increased the incidence of cutaneous tumours when compared with controls irradiated after treatment with vehicle. This effect was related to psoralen concentration and at the two concentrations investigated there was no significant difference between the compounds.

The psoralens are natural compounds which photoreact with DNA in the presence of UV-A radiation (315–400 nm). At the molecular level two types of DNA photoproduct have been reported—single strand adducts and interstrand crosslinks (Cole, 1970, 1971). Some psoralens, e.g. 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP), are capable of both types of reaction with DNA and are termed bi-functional but others, e.g. angelicin, form single strand adducts only and are called mono-functional. These reactions with DNA are the basis of a wide range of photobiological effects which include inhibition of DNA synthesis, mutagenesis and photocarcinogenesis (Scott, Pathak & Mohn, 1976). The precise roles of the different types of DNA-psoralen photoproduct are not known but the DNA crosslink is probably the more deleterious to the cell (Ben-Hur & Elkind, 1973; Coppey, Averbeck & Moreno, 1979; Babudri *et al.*, 1981).

Although psoralens photoreact with proteins (Yoshikama *et al.*, 1979) and also, when excited with UV-A radiation, produce singlet oxygen (De Mol, Beijersbergen van Henegouwen & van Beele, 1981), the significance of these reactions remains uncertain.

Psoralens (8-MOP) and UV-A radiation (PUVA) are now routinely used in the treatment of psoriasis (Parrish *et al.*, 1974). Ideally, a photochemotherapeutic agent should be clinically effective and without any long-term hazards, but the development of skin tumours in some

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PUVA-treated patients has prompted concern about the long-term safety of this treatment (Stern *et al.*, 1979).

Hönigsmann *et al.* (1979) have suggested the use of 5-MOP as an alternative to 8-MOP because the clinical efficacy of the former is associated with fewer side-effects, e.g. less skin erythema and oedema and less nausea when taken orally. However, 5-MOP has also attracted controversy as the active melanogenic ingredient of the bergamot (*Citrus bergamia*) oil that is added to several popular sunscreen preparations (Ashwood-Smith *et al.*, 1980).

8-MOP has long been established as a potent photocarcinogen when topically applied on rodent skin (Urbach, 1959; Forbes, Davies & Urbach, 1976), though not when given orally (Pathak *et al.*, 1959; Urbach, 1959), but there is only one published study on the photocarcinogenicity of 5-MOP (Zajdela & Bisagni, 1981).

Data on the relative photocarcinogenicity of psoralens with possible clinical and cosmetic uses are of obvious interest and value, even if related only to rodents and not directly to humans. In this paper we present data which compare the phototumorigenicity of 8-MOP and 5-MOP. We have used solar simulated radiation (SSR) and have taken particular care about radiometry.

MATERIALS AND METHODS

Animals

These were albino hairless mice, an outbred strain long established at the Institute of Dermatology, London. Each experimental batch comprised twenty males and twenty females which were kept in groups of five. Each mouse was identified with a small Indian ink tattoo at the base of its tail. At the start of each experiment, the mice were 8–10 weeks old. During the course of the experiments, animals were weighed once a week as a monitor of general health. Water and food (Dixon & Sons, FFG(M)) were allowed *ad libitum*.

Test substances

Crystalline 5-MOP and 8-MOP were provided by Laboratoires Goupil, France. Psoralen solutions were made up at 0·03% and 0·01% (w/v) in a vehicle of BP grade arachis oil (Hopkin & Williams) and isopropyl myristate 70:30 v/v. (The addition of the latter increased the solubility of 5-MOP). 5-MOP and 8-MOP showed the expected absorption spectroscopy and TLC characteristics and were found to be stable in the vehicle used.

Radiation source

This was a vertically mounted water-cooled 6 kW xenon arc (Wotan XBF 6000W/1) operated at 50 amp. The arc was at the centre of a lantern-like filter holder which was at a radial distance of 0·3 m to the arc. The filters (14·5 cm × 11 cm) were 2 mm thick Schott's glass WG320 (formerly called WG5). The resulting emission spectrum comprised ultraviolet, visible and infrared radiation. The UVR component, simulating solar radiation, is shown in Fig. 1.

Radiometry

Full details are described by Young *et al.* (1982). In summary, four methods were used: (a) indirect spectroradiometry, (b) an International Light IL 442A phototherapy radiometer, (c) an integrating UV-A radiation dosimeter, and (d) polysulphone film actinometry (Davis, Deane & Diffey, 1976). The estimated daily dose of ultraviolet radiation was about 1·7 J cm^{-2} of which about 40 mJ cm^{-2} was UV-B radiation. This exposure dose was determined empirically with

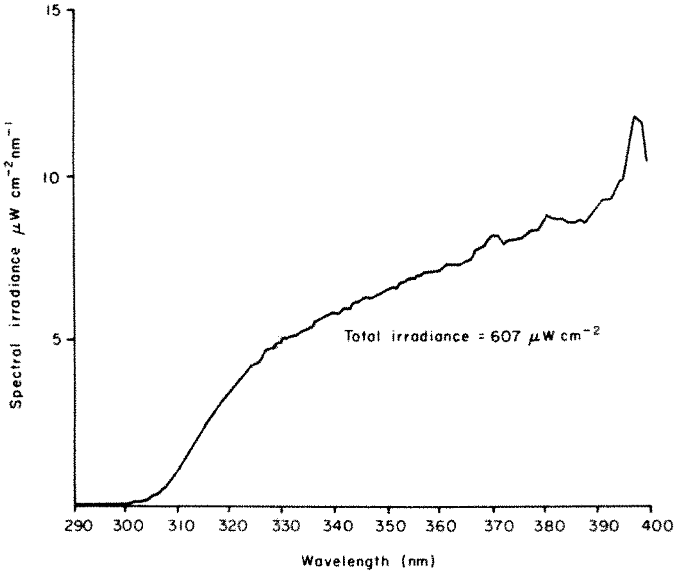

FIGURE 1. Emission spectrum of solar simulated radiation.

psoralens at the highest concentration (0·03%) as being about half the SSR dose required to produce an acute reaction. Daily exposure times were about 50 min.

Experimental procedure

After their flanks had been painted with the appropriate test substance, the mice were placed into cages, each with twelve individual compartments. The cages were hung from a circumferentially arranged scaffolding of 1·5 m radius to the arc. Animals could rotate during the exposure, but the dimensions of the individual units ensured that one or other flank faced the lamp. The irradiation was started approximately 30 min after skin painting. Five weekday exposures were given throughout the course of the experiments.

Tumour assessment

Animals were examined weekly for tumours, the longest diameters of which were measured and the location mapped on special charts. Tumours less than 1 mm in diameter were noted but not included in the tumour count. Tumours that had regressed or had been bitten off were still counted as tumours as long as they had previously attained a diameter of at least 1 mm. During the course of the experiments there was no evidence of tumour regression. Fig. 2 shows a mouse with several skin tumours. This animal was treated for 34 weeks with 5-MOP (0·03%) and SSR. At any one week, the number of tumours on different mice in the same treatment group varied widely.

Statistical analysis of data

Data analysis was done using two methods:

1) For graphic presentation of data, life tables were constructed using the actuarial (Kaplan-Meier) method recommended by Peto *et al.* (1980).

2) For the statistical comparisons between treatment groups, composite contingency table

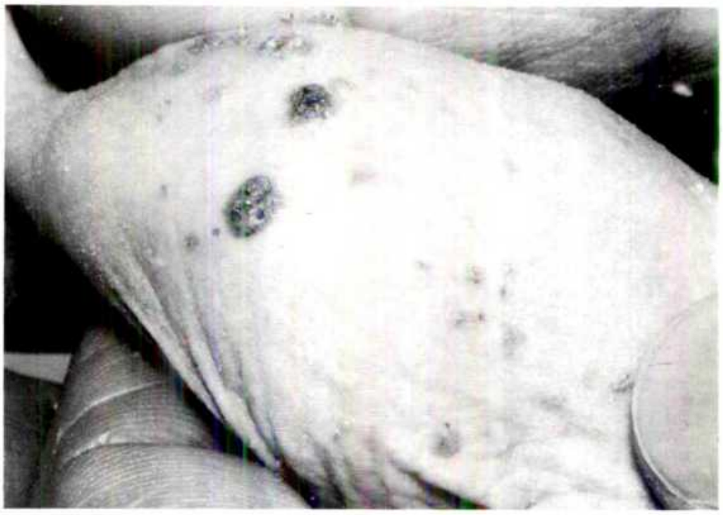

FIGURE 2. Posterior right flank of mouse showing tumours.

methods were used as described by Peto *et al.* (1980). These methods have the advantage of being sensitive, relatively simple to use and model-free.

When more than two groups were being compared, P-values were based on a one-tailed test for positive trend with respect to psoralen concentration (0%, 0·01% and 0·03%), as recommended by Peto *et al.* (1980). When only two groups were being compared (e.g. 5-MOP vs. 8-MOP), two-tailed P-values were used.

Tumour histology

When 100% of animals treated with 8-MOP and 5-MOP at 0·03% had developed tumours, a random sample (computer generated) of seventy-six of these tumours was taken for standard histology. Sections from paraffin blocks were stained with haematoxylin and eosin and examined with a light microscope. Similarly, sixteen tumours were taken from the 0·01% 8-MOP group when tumour incidence had reached 100%. A further nineteen tumours were taken from this group 12 weeks later, during which time the animals had no experimental treatments. Tumours were not classified as carcinomas unless the panniculus carnosus had been invaded. The group of tumours classified as 'borderline' did not invade this muscle but showed marked atypicality of individual keratinocytes and/or frequent mitotic figures.

RESULTS

Animals treated with SSR

Figs 3 and 4 show the results with two different concentrations of 8-MOP and 5-MOP respectively. Both psoralen compounds markedly increased the onset rates of tumours, since for both a statistical test of the data (displayed in Figs 3 and 4) revealed a highly significant positive trend with respect to increased psoralen concentration (one-tailed $P < 0{\cdot}01$). At 0·03% the onset ratio, viz. (observed number of animals with tumours)/(expected number of animals with tumours), was higher with 8-MOP. However, the difference in photocarcinogenic potential of

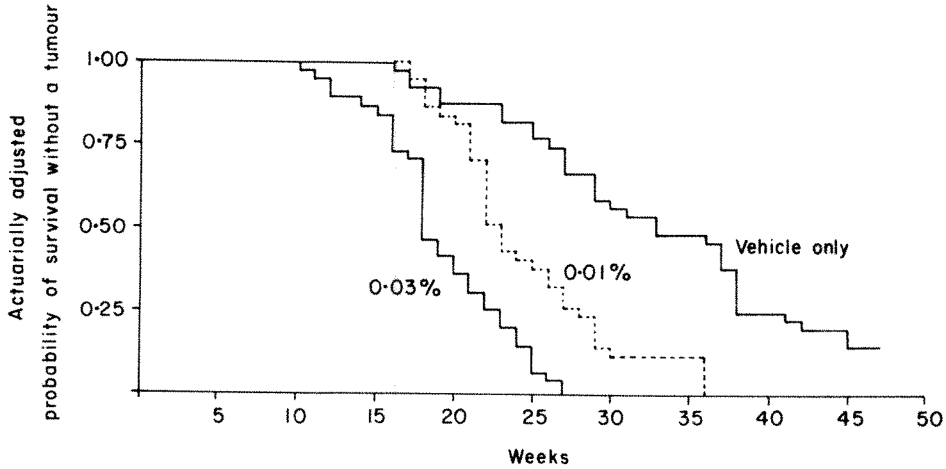

FIGURE 3. Life-tables of mice treated with 8-MOP and SSR.

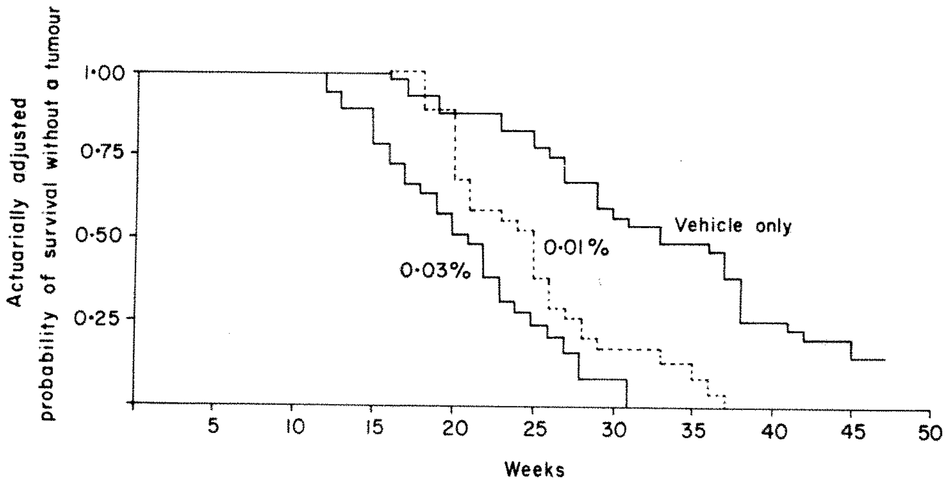

FIGURE 4. Life-tables of mice treated with 5-MOP and SSR.

the two compounds was not statistically significant (one-tailed $P > 0{\cdot}05$). Details of this analysis are shown in Table I.

Fig. 5 compares the effects of SSR alone with those of vehicle plus SSR. Treatment with base significantly increased cancer incidence over the whole period of the experiment (2-tailed $P < 0{\cdot}01$). For this reason, it is appropriate to compare the psoralen-treated animals only with the vehicle-treated controls.

Control studies without SSR

Results are compared at 26 weeks as this was the minimum period of all experiments. In the limiting experiment arachis oil only was used.

Animals that had no treatment other than being put in cages every day had a 0·95 probability

TABLE 1. Photocarcinogenic potential of 8-MOP and 5-MOP. Combined data analysis of comparison of 8-MOP plus SSR with 5-MOP plus SSR at two psoralen dose levels. Treatments are equivalent because Σy (7·3 ± s.d. 5·3) differs only randomly from zero (two-tailed $P > 0·05$), as expected if "null" hypothesis is valid

Dose Level	Period of Comparison (weeks)	8-MOP			5-MOP			
		O	E	y	O	E	y	v
0·03%	27	38	31·4	6·6	26	32·6	−6·6	13·89
0·01%	36	36	35·3	0·7	31	31·7	−0·7	13·91
	Σ	74	66·7	7·3	57	64·3	−7·3	27·80

O = observed number of mice with tumours; E = expected number of mice with tumours; $y = (O - E)$; v = variance of y; s.d. of $\Sigma y = \sqrt{\Sigma v}$.

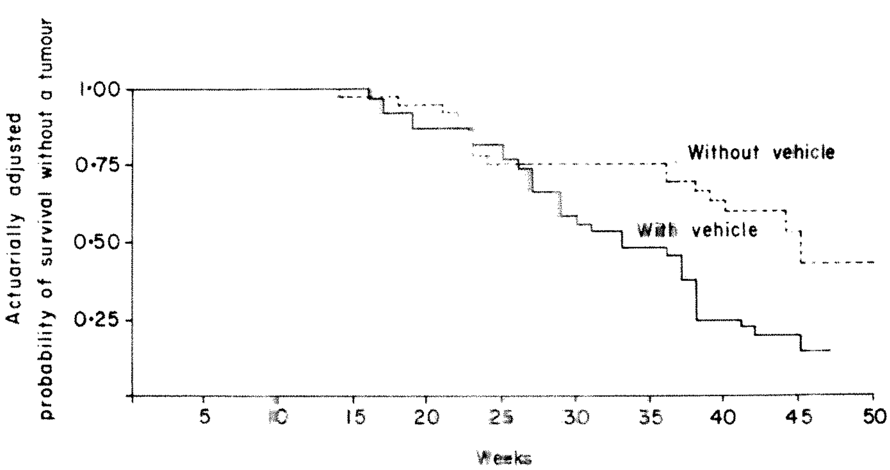

FIGURE 5. Life-tables of mice treated with SSR alone and SSR plus vehicle.

(actuarially adjusted) of remaining tumourless at 26 weeks. When treated with vehicle (arachis oil and isopropyl myristate) before being caged the corresponding probability was 0·97. These numbers clearly do not differ significantly. One group of animals was treated with 100% arachis oil only and then caged. At 26 weeks the probability (actuarially adjusted) of remaining tumourless was 0·85, but even this was not statistically significantly different (two-tailed $P > 0·05$) from the other two control groups described above.

At 26 weeks the corresponding probabilities in unirradiated animals treated with both vehicle (arachis oil and iospropyl myristate) and psoralens at 0·03%, were 0·84 for 8-MOP and 0·85 for 5-MOP. There was no significant difference between these two groups or between these two groups and any of the above three groups (two-tailed $P > 0·05$).

Table 2 provides an ancillary analysis of the mean number of tumours per surviving mouse at 26 weeks for all the groups described above. It is apparent that treatment with psoralens plus

SSR increases tumour numbers as compared with controls but it must be remembered that such means may be unreliable, as some of them are strongly dependent on a few individual animals that developed very large numbers of tumours.

Table 3 shows the result of the histological analysis of all the lesions in the psoralen experiments that were sectioned. It can be seen that with both compounds most of the tumours were squamous cell papillomas, as indeed were most of the tumours among the controls.

TABLE 2. The mean number of tumours per surviving mouse at 26 weeks

Group	Total no. of tumours / no. of surviving mice	Mean no. tumours/mouse
No treatment	2/40	0·05
Vehicle alone	1/31	0·03
Arachis oil alone	8/39	0·2
SSR alone	18/33	0·5
Vehicle plus SSR	14/39	0·4
8-MOP (0·03%) alone	6/26	0·2
5-MOP (0·03%) alone	5/31	0·2
8-MOP (0·03%) + SSR	372/38	9·8
5-MOP (0·03%) + SSR	179/31	5·8
8-MOP (0·01%) + SSR	69/36	1·9
5-MOP (0·01%) + SSR	84/34	2·5

SSR = Solar simulated radiation.

TABLE 3. Histological analysis of lesions

	Psoralen (0·03%) + SSR		8-MOP (0·01%) + SSR	
	8-MOP	5-MOP	End of irradiation	12 weeks post-irradiation
Squamous papilloma	84%	83%	81%	74%
Borderline	3%	8%	0%	5%
Invasive squamous carcinoma	3%	0%	18%	21%
Not tumour	11%	10%	0%	0%

DISCUSSION

These results confirm and extend earlier studies on the phototumorigenesis by psoralens in laboratory animals. The present study appears to be the first of its kind to examine the effect of psoralen dose.

Control experiments

Several different types of control experiments were run. The prevalence of spontaneous skin tumours in unirradiated animals was low, and among them no clear effects were evident with vehicle alone or with vehicle plus psoralen. This suggests that the effects of the psoralens are strictly phototumorigenic.

Furthermore, up to about 26 weeks, there was no apparent difference between the results of treatment with SSR alone and treatment with topically applied vehicle *plus* SSR. However, over the whole period of the experiment of about 45 weeks, treatment with vehicle alone significantly enhanced the tumorigenicity of SSR. The reasons for this slightly surprising result are not known and replication would be desirable. It is possible that (a) the vehicle merely alters UV-B radiation transmittance, or that (b) the vehicle itself is mildly tumorigenic or phototumorigenic, or (c) that a stress factor may be involved with the application of the vehicle (Riley, 1981).

Phototumorigenic potential of the test agents

With SSR, both 8-MOP and 5-MOP show a highly significant dose-response effect, but they are not significantly different from each other in their phototumorigenic potential.

The comparable efficacy of these two compounds in this study is not surprising in view of the similarity of their molecular structures and photochemical and photo-biological properties. This quantitative similarity suggests that under identical conditions of use (and assuming similar metabolism of both compounds) they would have similar phototumorigenic potential in man.

Differences between this and other studies

In a similar comparative study in which the ears of haired mice were exposed to 365 nm radiation, Zajdela & Bisagni (1981) reported both compounds to be phototumorigenic but with very much longer latent periods and tumour development times than shown here. These dissimilarities are probably related to differences in experimental protocol, viz., to differences in anatomical site, mouse strain and spectral output of irradiation sources (Grube, Ley & Fry, 1977). The French workers used acetone as a vehicle for the psoralens instead of arachis oil/isopropyl myristate. Although the concentration of topical psoralens applied by Zajdela & Bisagni (1981), viz. 0·025%, was similar to ours, penetration into the skin may be different as epidermal penetration of psoralens seems to be strongly influenced by vehicle (Kammerau *et al.*, 1976). Furthermore, with acetone, which is very volatile, psoralen recrystallization was observed on the stratum corneum (Zajdela & Bisagni, 1981). The arachis oil/isopropyl myristate mixture which visibly persists on the skin for about half an hour may allow better penetration of psoralens. In dosimetric terms the daily UVR exposure given by Zajdela & Bisagni (1981) was also similar to ours, but probably lower in terms of biological reactivity. This may be related to the relatively poor ability of 365 nm radiation to induce DNA crosslinks (Fujita, Sano & Suzuki, 1979) and other biological endpoints, as compared with the waveband 320–335 nm which is particularly potent for many psoralen photobiological effects (*see* Young & Magnus, 1981). However, the action spectrum for psoralen phototumorigenesis remains unknown. Another difference between our studies and those of Zajdela & Bisagni (1981) was that the latter found 8-MOP a more potent photocarcinogen than 5-MOP. The reason for this difference is not clear but it may also be related to psoralen recrystallization on the skin surface and/or differences in the solubility of 5-MOP and 8-MOP (the former is much less soluble in organic, aqueous and lipid solvents).

Among irradiated mice receiving 0·03% 8-MOP or 5-MOP, tumours taken for microscopic examination at 31 weeks, i.e. when 100% of animals had tumours, were mostly squamous cell papillomas. A similar pattern was observed when tumours were taken at the end of irradiation (at 36 weeks) or at 12 weeks thereafter in the 0·01% 8-MOP experiment. In contrast, other workers including Zajdela & Bisagni (1981) and Forbes *et al.* (1976) have reported a high percentage of squamous cell carcinomas. The small proportion of invasive tumours observed in this study may be due to: (1) the relatively short time-course of the present experiments for, in purely chemical carcinogenesis studies carcinomas may develop after some time from a benign lesion; (2) the strain of animals; (3) the anatomical site; or (4) some unknown effect when an oily vehicle is used.

Possible mechanisms of psoralen photocarcinogenesis

Reactions with DNA. UV-A radiation causes covalent bonding between psoralens and DNA. Crosslinks and two types of mono-adduct may be formed, viz. 3,4 and 4′,5′ mono-adducts. The biological significance of these reactions is not fully understood. Both mono-adducts and crosslinks are mutagenic (Bridges, Mottershead & Knowles, 1979; Scott & Maley, 1981) but it seems likely that the crosslink is the more lethal lesion (Ben-Hur & Elkind, 1973; Seki, Nozo & Kondo, 1978; Babuduri *et al.*, 1981). Bi-functional psoralens induce sunburn cells (cell shrinkage necrosis) in mouse epidermis more readily than do mono-functional psoralens (Young & Magnus, 1982).

There are no definitive data on the roles of the different DNA lesions in psoralen photocarcinogenesis. An investigation by Grube *et al.* (1977) indicated no clear relationship between DNA crosslinking and tumorigenesis in the hairless mouse. Psoralen (bi-functional) is more effective than angelicin (mono-functional) at inducing micronuclei in mammalian cells *in vitro* (Ashwood-Smith *et al.*, 1977), suggesting that crosslinks may be more effective than mono-adducts at inducing large-scale changes in the genome that Cairns (1981) has suggested may be particularly carcinogenic. The mono-functional 3-carbethoxypsoralen has been reported as being non-photocarcinogenic in the mouse (Dubertret *et al.*, 1979) but this compound is very photolabile and so may not be suitable for assessing the role of mono-adducts in photocarcinogenesis. In contrast, another mono-functional compound, 4,5′ dimethylangelicin (Bordin *et al.*, 1979) is highly photocarcinogenic (M. Pathak, personal communication). Quantitative aspects, as well as qualitative ones, may also be important, viz. the number of different types of unrepaired lesions per 1000 nucleotides. Even with the carcinogenic bi-functional psoralens most of the DNA lesions *in vitro* are mono-adducts of which only the less common 4′,5′ type can be converted into crosslinks (Dall'Acqua *et al.*, 1979a,b).

Normal human fibroblasts readily repair thymine dimers induced by UV-C radiation but where this repair is defective, as in patients with xeroderma pigmentosum (XP), the consequence is a very high prevalence of UVR-related skin cancers (Cleaver, 1969). *In vitro* fibroblasts from XP patients are unable to repair psoralen damage compared with normal fibroblasts (Baden *et al.*, 1972; Kaye, Smith & Hanawalt, 1980), and XP patients given psoralen therapeutically in an attempt to induce a prophylactic skin tan, seemed to develop skin tumours more quickly (Reed, 1976). This suggests that a lack of repair of psoralen damage to DNA may have been critical. In normal cells, crosslinks are less readily repaired than mono-adducts (Kaye *et al.*, 1980).

The molecular basis of psoralen photocarcinogenesis remains unestablished. If the crosslink is more likely to lead to individual cell death, e.g. a sunburn cell, than the mono-adduct, the former lesion may be of less consequence to the organism. On the other hand, if the crosslink is inadequately repaired, but none the less permits cell survival, this lesion may be the more serious.

There appears to be a positive linear correlation between lifespan and the efficacy of excision repair of 254 nm-induced DNA damage in fibroblasts of seven mammalian species (Hart &

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Setlow, 1974). Thus, human, elephant and cow fibroblasts are relatively efficient at excision repair compared with mouse and hamster cells which have a repair capacity about ten times lower. Comprehensive comparative studies of psoralen repair in different species have yet to be done. The ratio of superoxide dismutase activity to metabolic rate is also positively correlated with lifespan (Tolmasoff, Ono & Cutler, 1980). These data suggest that short-lived species may be more sensitive to environmental radiation hazards and therefore quantitative extrapolation of photocarcinogenicity studies in rodents to man is not reliable (Peto, 1979). A further difference in albino animals is their inability to develop the skin melanin which in man might be expected to afford at least some protection against further UV-B radiation assault.

A role for the immune process. Kripke (1980) has reviewed the increasing evidence for the role of immunological factors in skin cancer induced by UV-B radiation. These include alteration of the antigen presenting function of Langerhans cells and the activation of suppressor T lymphocytes.

PUVA treatment has been reported to have both local and systemic immunosuppressive effects in man and in experimental animals (Strauss *et al.*, 1980; Bridges *et al.*, 1981; Morison *et al.* 1981), and psoriatics taking PUVA therapy show a marked decline of Langerhans cell counts (Friedmann, 1981). Examination of the psoriatic follow-up data of Stern *et al.* (1979) has led Bridges & Strauss (1980) to postulate that PUVA may act as a 'pseudo' promoting agent of skin cancer by depressing possible immunological surveillance mechanisms and thus allowing lesions initiated by other agents, e.g. X-rays, to be expressed.

Some support is lent to this theory by data from Kinlen *et al.* (1979) who reported a striking increase of squamous skin cancer in immunosuppressed, sun-exposed, Australian patients. Furthermore, both 5-MOP and 8-MOP induce ornithine decarboxylase, in mouse skin exposed to UV-A radiation (Lowe *et al.*, 1982), which may be related to skin tumour promotion (O'Brien, 1976).

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Topical 8-methoxypsoralen photochemotherapy of psoriasis: a clinical study

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SUMMARY

Topical psoralen plus UV-A irradiation (topical PUVA) was re-evaluated with regard to the timing of UV-A exposure. Symmetrical lesions of fifteen patients with psoriasis were treated with topical PUVA. One side was exposed to UV-A 2 h after topical application of 1% 8-methoxypsoralen (2-h interval therapy), while the other side was exposed to the same dose of UV-A within 5 min of the topical application (non-interval therapy). Both regimens were effective. The non-interval therapy was preferred in one case, the 2-hour interval was preferred in three cases, and there was no clear difference in the other eleven cases. There was less tendency to burning with the non-interval therapy, and our study suggests that this is a useful regimen in the out-patient treatment of psoriasis.

Oral or topical psoralen compounds plus long-wave ultraviolet radiation (PUVA) have been successfully used in the treatment of psoriasis. In the case of oral PUVA, UV-A irradiation is appropriately given 2 h after oral ingestion of the psoralen when the plasma level reaches its peak (Ljunggren, Carter & Albert, 1980), but the treatment schedule for topical PUVA has not yet been standardized. Many clinicians allow 1–2 h to elapse between the topical application of the psoralen and UV-A exposure, but our preliminary report has shown that topical 8-methoxypsoralen (8-MOP) followed immediately by UV-A irradiation can also clear psoriatic lesions (Danno *et al.*, 1982). We have now performed a controlled trial to evaluate the therapeutic applicability of this non-interval regimen.

METHODS

Subjects

Fifteen patients with chronic, stable and extensive psoriasis, 20 to 78 years old (mean age 53·8),

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were treated with topical PUVA. Thirteen patients suffered from plaque psoriasis (mild in four, severe in nine) and two patients were erythrodermic.

Psoralen

A 1% solution of 8-MOP (Oxsoralen® lotion) was commercially available from Taisho Pharmaceutical Company (Tokyo, Japan). The base of this solution consists of a mixture of ethanol, acetone and propylene glycol (7:2:1 by volume).

Light source

We used a cabinet containing seventy-two fluorescent black light tubes (Toshiba FL 20S BLB) with aluminium reflectors. The tubes are arranged so as to cover two aspects of the body (thirty-six tubes each on the top and at one side). The total energy output of this cabinet was 8·5 mW/cm^2 with a distance of 30 cm. Two-minute exposure gave approximately 1·0 J/cm^2.

Determination of minimal phototoxic dose (MPD)

Before PUVA therapy, an uninvolved area of the back of each patient was painted with 8-MOP and irradiated with various amounts of UV-A after a 2-h interval and the erythematous response was examined 2 days later. The minimal dose producing visible erythema was determined as a MPD.

PUVA treatment

In each patient symmetrical areas, mainly on the trunk, were chosen as the treatment sites. One side was irradiated with UV-A light 2 h after the painting with Oxsoralen® lotion (approximately 10 μl/cm^2) (2-hour interval therapy), while the other site was irradiated as soon as possible (within 5 min) after the drug was applied (non-interval therapy). UV-A dosimetry was principally based upon the MPD. Patients received exposures principally three times a week (two times a week in case 12) and dose adjustments were individualized according to the patients' MPD and the response of the psoriatic lesions. Three patients received PUVA therapy combined with topical corticosteroid therapy. The latter was applied daily or on alternate days (cases 2, 9 and 10).

Evaluation of therapeutic responses

Therapeutic responses to PUVA were evaluated as follows: 0 indicates pre-treatment or no response; 1+ diminished scaling; 2+ decreased erythema and infiltration; 3+ minimal infiltration; and 4+ complete clearing.

RESULTS

Details of the treatment data are summarized in Table 1. The MPD ranged from 0·5 to 2·5 J/cm^2 (mean dose 1·4 J/cm^2). Initial exposure doses were between 0·2 and 2·5 J/cm^2 (mean dose 1·0 J/cm^2) (1/2 to 1 MPD) and final exposure doses were between 0·25 and 3·6 J/cm^2 (mean dose 1·4 J/cm^2). Seven to 26 exposures (mean 16) were totalled during 3 to 10 weeks (mean 5·5 weeks). The cumulative energy doses ranged from 4·5 to 36·2 J/cm^2 (mean dose 16·0 J/cm^2). At the completion of the treatments, complete clearing (4+) or marked improvement (3+) was achieved in many cases with both the non-interval or the 2-hour interval therapy. Two patients (cases 2 and 13) receiving a short term PUVA treatment gained only partial improvement.

Comparison of efficacy between the two treatment regimens revealed that (1) the non-interval

TABLE 1. Summary of the treatment data with non-interval therapy (TI) and 2-hour interval therapy (TII)

Case/Age/Sex	Type*	Treatment sites†	MPD‡ (J/cm^2)	Exposure dose (J/cm^2)		No. of treatments	Duration of treatment (weeks)	Total exposure dose (J/cm^2)	Final therapeutic response (TI/TII)	Additional therapy	Side-effects
				Initial	Final						
1/50/F	ER	B	2·5	2·5	2·5	16	6	36·2	3+/2+		burn (TII)
2/73/F	NM(s)	B,CW	2·5	2·0	2·0	7	3	11·0	2+/3+	Topical C	
3/73/F	NM(s)	B	1·0	0·5	1·0	17	5	10·0	2+/4+		
4/78/M	NM(m)	B	0·5	0·25	1·0	20	6	15·0	3+/4+		burn (TII)
5/60/F	ER	B	0·5	0·2	1·3	26	8	20·8	3+/3+		burn (TII)
6/45/M	NM(s)	B,CW	0·5	0·25	0·25	20	6	5·0	4+/4+		
7/77/F	NM(s)	B	2·0	0·75	0·75	20	7	15·0	4+/4+		
8/65/M	NM(s)	B,CW	1·5	0·75	0·75	17	5	10·5	3+/3+		
9/40/M	NM(s)	B	1·5	1·5	1·5	15	5	19·5	3+/3+	Topical C	
10/48/M	NM(s)	B	1·0	0·75	1·75	21	7	23·3	3+/3+	Topical C	
11/51/M	NM(s)	B	1·5	1·0	1·0	15	4	15·0	4+/4+		
12/56/M	NM(s)	B,BT	2·0	1·2	3·6	19	10	22·5	4+/4+		burn (TII)
13/26/F	NM(m)	B,CW	0·5	0·5	1·0	8	3	6·0	2+/2+		
14/45/M	NM(m)	B,AW	0·5	0·25	0·75	9	3	4·5	3+/3+		
15/20/M	NM(m)	B	2·5	2·5	2·5	10	4	25·0	4+/4+		
Average			1·4	1·0	1·4	16	5·5	16·0			

*ER: erythrodermic type, NM: nummular type, m: mild form, s: severe form
†AW: abdominal wall, B: back, BT: buttocks, CW: chest wall
‡MPD: minimal phototoxic dose
§C: corticosteroid

therapy was preferred in one case (case 1); (2) the 2-hour interval therapy was preferred in three cases (cases 2, 3 and 4); and (3) both regimens were equally effective in the other eleven cases (cases 5 to 15).

In patient 1, erythema and scaling began to diminish after five exposures with both regimens. After nine exposures, the lesions improved with the non-interval therapy in advance of the other. By sixteen exposures the non-interval therapy gave marked improvement (minimal infiltration was noted), while erythema and infiltration still remained in the areas treated with the 2-hour interval.

In the second group of patients (cases 2, 3 and 4), erythema, scaling and infiltration began to diminish after five to six exposures with both treatment regimens. Thereafter, the lesions responded more promptly to the 2-hour interval therapy than to the non-interval therapy. In cases 3 and 4, complete clearing was finally achieved with the 2-hour interval therapy, while psoriatic lesions were resistant to the latter regimen, leaving visible erythema and infiltration.

In the last group of the patients (cases 5 to 15), psoriatic lesions appeared to respond more favourably to the 2-hour interval therapy than to the other during the first five to ten exposures, as was the case in the second group. In these patients, however, continuation of the non-interval therapy finally gave results as good as in the 2-hour interval therapy. Initial clearing or appreciable improvement was obtained with either method. A representative case (case 12) is shown in Fig. 1 (a, b and c).

With the 2-hour interval, overdose of UV-A occasionally caused burning after several exposures (cases 1, 4, 5 and 12), while the same amounts of UV-A without an interval did not produce a burn. Some of the patients receiving the non-interval therapy complained of irritating sensations in the treatment areas (cases, 1, 8, 9 and 10) but this side-effect was transient and tolerable. Burning was relieved by reducing the UV-A dose and by topical application of corticosteroids. The patients receiving suberythemogenic doses throughout the treatment period achieved complete clearing or notable improvement without unfavourable side-effects (cases 2, 6, 7, 8 and 11). In these patients pigmentation following multiple treatments was found to be milder.

After the comparison was completed, the majority of the patients continued to receive maintenance therapy once a week for up to 10 months with either the non-interval (cases 1 and 8) or the 2-hour interval method (other cases). No recurrence has so far occurred with either method.

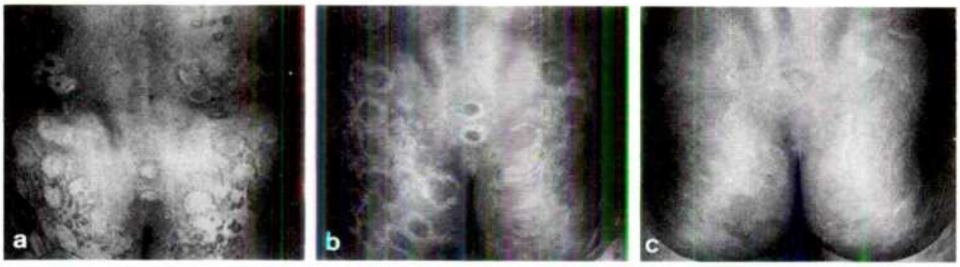

FIGURE 1. Topical photochemotherapy of psoriasis. The left side of the back and buttocks was treated for a 2-hour interval, while the right side was treated with the non-interval method (case 12). (a) Before treatment; (b) after 10 exposures. The lesions appeared to respond more favourably to the 2-hour interval therapy (left side) than to the other (right side); and (c) after 18 exposures. Complete clearing was achieved with both methods.

DISCUSSION

The results of our study suggest that the non-interval therapy with topical PUVA is therapeutically acceptable in the treatment of psoriasis. The non-interval therapy seems to be of advantage both to patients and clinicians because they do not waste time. In other laboratories, UV-A exposures have been given as soon as 10 min after topical application of psoralens with reported success (Tronnier & Schüle, 1973). Another merit of the non-interval therapy was that unfavourable side-effects were minimal, and it is easy to treat patients as out-patients.

Petrozzi, Kaidbey & Kligman (1977) demonstrated that the optimal time interval for production of the maximum phototoxic reaction, i.e. visible erythema, was 2 hours. We confirmed their observations and found that with no interval UV-A exposure produced minimal erythematous reactions (Danno *et al.*, 1982). However, this PUVA regimen was still able to clear psoriatic lesions, suggesting that phototoxic reactions may not be relevant to the therapeutic response. Kenicer *et al.* (1981) also reported a satisfactory clinical response to low output PUVA (total exposure dose 21·3 J/cm^2) in approximately one third of the patients treated. Furthermore, low-dose therapy would be less carcinogenic.

The mode of action of the non-interval therapy is obscure. However, we can offer the following speculations. First of all, it has been shown that psoralen compounds penetrate into the epidermis rapidly (within 10 min after the painting) in normal human skin (Kammerau *et al.*, 1976) and, presumably, more rapidly in psoriatic lesions. Therefore, the penetrated drug would make photoadducts with DNA molecules even if UV-A light is given as soon as the drug is painted, resulting in significant inhibition of epidermal DNA synthesis (Danno *et al.*, 1982). Even with suberythemogenic doses of UV-A light DNA synthesis was notably suppressed.

Secondly, PUVA may act by its effects on the immune system including lymphoid cells (Morison, Parrish & Epstein, 1979) and psoriasis leukotactic factors (PLF) (Mizuno, Enami & Esaki, 1979). Since PLF are present in the lesional horny layer and psoralens would reach there shortly after they are applied, the non-interval therapy is likely to inactivate them to prevent accumulation of polymorphonuclear leukocytes to the lesions. Finally, since we used a relatively high concentration of 8-MOP, it is possible that sufficient amounts of the drug remained in the affected skin from one treatment to the next.

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Stimulation of pigmentation in melanoma cells by trimethylpsoralen in the absence of ultraviolet irradiation

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SUMMARY

The effects of trimethylpsoralen and 8-methoxypsoralen on the pigmentary characteristics of cultured murine melanoma cells were investigated in the absence of ultraviolet irradiation. Stimulation of pigmentation was assayed by gross examination of centrifuged cell pellets, light microscopy of cells, electron microscopy, a biochemical determination of cell tyrosinase activity, and a chemical determination of cell melanin content. It was found that an adequate concentration (10^{-5} M) of trimethylpsoralen for an adequate exposure time stimulated pigmentation in the dark, whereas 8-methoxypsoralen in the same experimental setting did not stimulate pigmentation.

For thousands of years naturally occurring psoralens have been used in conjunction with sunlight to stimulate repigmentation in vitiligo (Pathak, Kramer & Fitzpatrick, 1974); however, even quite recently, *in vitro* studies of psoralen effects on pigment cells have been relatively scarce. Carter *et al.* (1974) reported the stimulation of tyrosinase activity of Cloudman melanoma cells by trimethylpsoralen (TMP) and long wave ultraviolet radiation (UV-A). Carter, Pan & Varga (1979) reported stimulation of tyrosinase activity in the same system with both TMP and 8-methoxypsoralen (8-MOP) in conjunction with UV-A. No stimulation of tyrosinase activity occurred in the absence of UV-A in either of the above studies.

The purpose of the present study was to determine whether adequate concentrations of psoralen over a long enough time would stimulate pigment production in the absence of UV-A irradiation.

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METHODS

Culture conditions

A cloned line of B16 murine melanoma cells was kindly provided by Takuji Takeuchi, D.S. of Tohoku University, Sendai, Japan. Cells were grown as monolayers in Minimum Essential Medium with Earle's Salts and glutamine (Gibco). Medium was supplemented with 10% (v/v) fetal bovine serum, 100 u/ml penicillin, 100 u/ml streptomycin, and 2% (v/v) 1 M Hepes buffer (Gibco).

25 cm^3 flasks (Costar) containing the above medium were inoculated on day 0 with 1×10^6 cells. On day 1, medium was replaced with that containing 10^{-7}–10^{-5} M concentrations of psoralens, and psoralen-containing media were changed on days 5, 9, and 13. On day 14, the cells were examined for colour changes. Cells were harvested from the flasks with 0·025% trypsin and 0·025% EDTH in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (BSS). Cells were counted with a haemocytometer in order to normalize all data from the assays. The cells were then centrifuged at 2000 r.p.m. for 10 min. The supernatant was decanted and the cells were resuspended in Ca^{2+} and Mg^{2+}-free Hank's BSS. These were again centrifuged, the supernatant was decanted and the cell pellets were stored at −20°C for no more than 3 days until the time of the assay.

In addition, using the same procedure, cells were grown in flasks for electron microscopic studies. The cells were fixed in the flasks at day 14 with glutaraldehyde. They were removed from the flask with a small rubber 'policeman', centrifuged, post-fixed in osmic acid, dehydrated, and imbedded in Epon. Thin sections were prepared and stained with uranyl acetate and lead citrate. Transmission electron photomicrographs were made using a RCA EMU 4C electron microscope.

It must be emphasized that in all experiments cells were grown under light-proof conditions. Flasks were at all times wrapped in light-proof aluminium foil. During experimental manipulations, the only light sources were a small incandescent bulb and a bunsen burner. UV-A radiation from both of these sources was looked for using an IL 442A UV-A phototherapy radiometer. None was detected from either source.

Psoralens

8-methoxypsoralen (MOP) and trimethylpsoralen (TMP) were obtained from Sigma Chemical Company. Stock solutions (10^{-2} M) in dimethylsulphoxide (DMSO) were prepared and glass-filtered for sterility. Ten-fold dilutions of this stock solution were prepared in the medium to final concentrations of 10^{-5}, 10^{-6} and 10^{-7} M. All experiments were performed using these concentrations in quadruplicate. Control studies were also performed in quadruplicate at various concentrations of DMSO corresponding to the concentrations in the experimental flasks (0·1%, 0·01% and 0·001%).

Tyrosinase activity

Cell pellets were assayed for tyrosinase activity by the method of Pomerantz (1969) as modified by Pawelek (1976). Pellets were treated with a detergent (Triton-X) to lyse the cells. An aliquot of lysate was then allowed to react with 3,5 tritiated L-tyrosine (New England Nuclear) in the presence of 0·005% L-Dopa in 1 M phosphate buffer at pH 6·8. The reaction proceeded for 30 min at 37°C at which time 10% activated charcoal in 0·1 M citrate was added to stop the reaction. The reaction mixture was then passed through a column of Dowex-50. Tyrosinase activity was assayed by the amount of tritiated water that was present in the reaction mixture which was

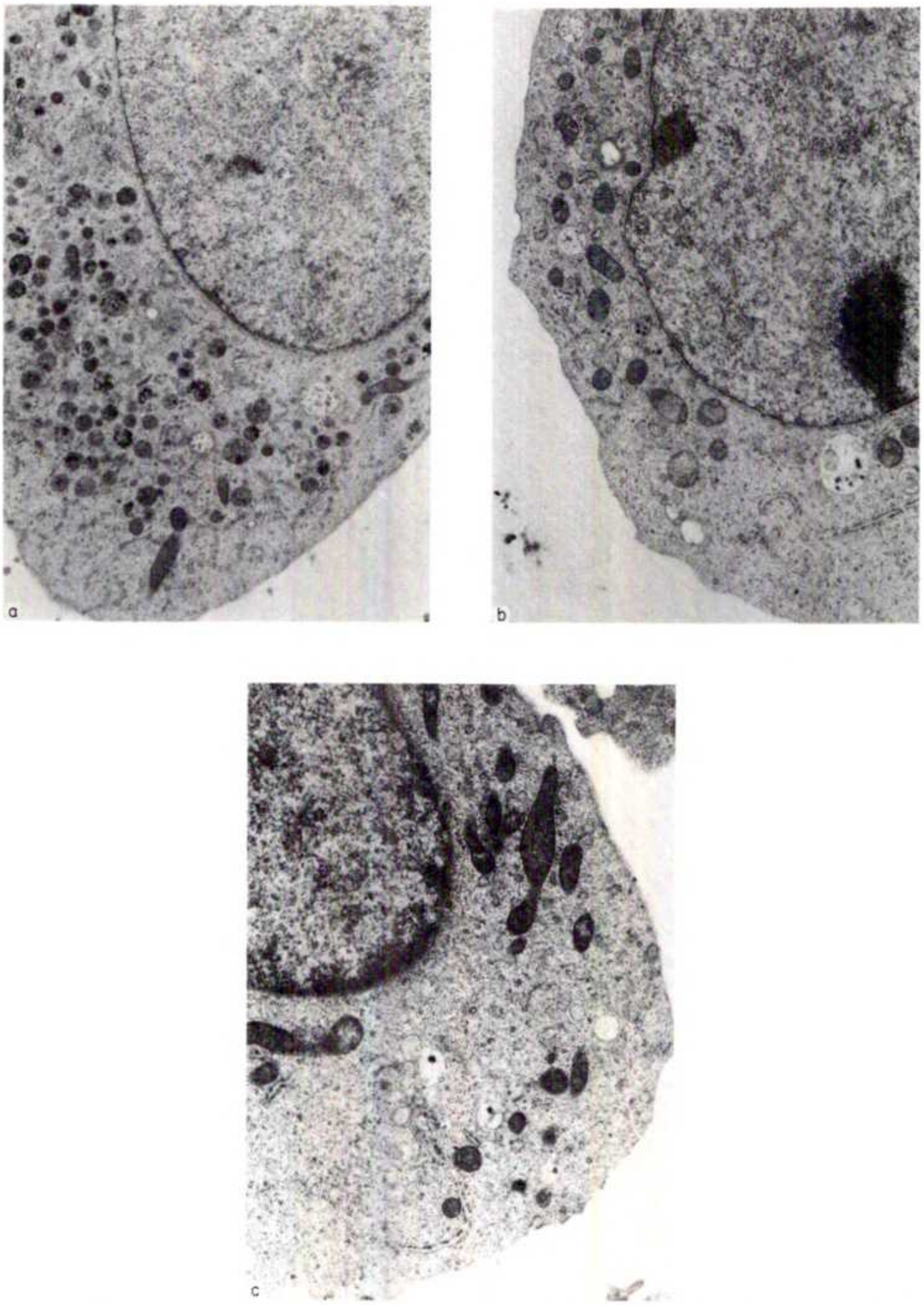

FIGURE 1. Transmission electron micrographs of murine melanoma cells. (a) treated with trimethylpsoralen (× 5300). (b) treated with 8-methoxypsoralen (× 9600). (c) control (× 5300).

produced by the release of tritium from the labelled tyrosine catalyzed by the cell tyrosinase. This was measured by scintillation counting and expressed as counts per minute per cell.

Cell melanin content

Cell pellets were assayed for melanin content by the method of Whittaker (1968) as modified by Wrathall *et al.* (1973). Cell pellets were subjected to three serial extractions with 5% trichloracetic acid, two serial extractions with ether/ethanol (1:3) and one extraction with absolute ether. The air-dried residue was dissolved in 0·85 M sodium hydroxide and heated for 10 min at 100°C. After cooling, optical density was measured at 400 nm with a Gilford spectrophotometer, Model 250. Melanin content was expressed as optical density (*D*) per cell.

RESULTS

Visual examination of media decanted from flasks after millipore filtration revealed slight but appreciable changes. Media from those cells treated with 10^{-5} M concentrations of TMP were darker than the media from the control flasks. Media from cells treated with MOP did not demonstrate a darkening effect.

Examination of washed cell pellets revealed similar results. Those cells treated with 10^{-5} M TMP showed a brownish darkening that was absent in both the control cells and those cells treated with MOP.

Phase contrast light microscopic examination of live cell monolayers revealed increased cytoplasmic darkening and granularity in those cells treated with 10^{-5} M TMP as compared with control cells. As above, those cells treated with MOP did not show cytoplasmic changes when compared with controls.

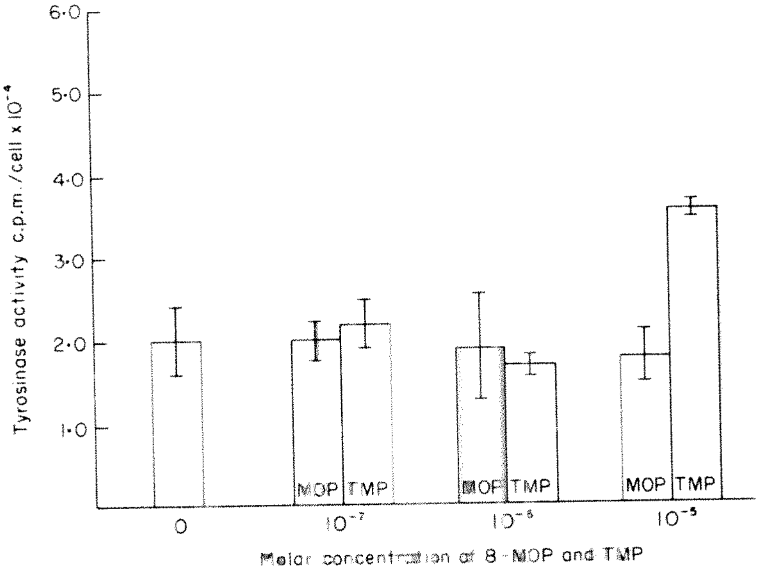

FIGURE 2. Tyrosinase activity vs. molar concentration of 8-methoxypsoralen (MOP) and trimethylpsoralen (TMP). Bars indicate standard deviations.

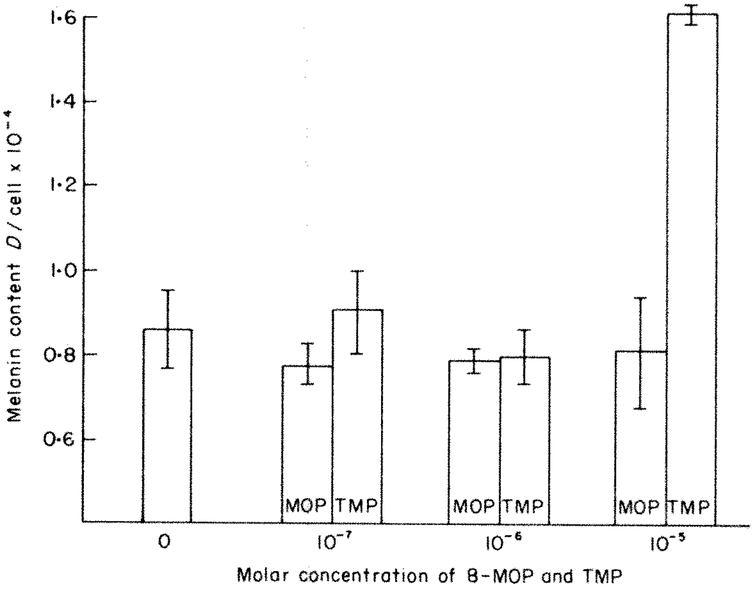

FIGURE 3. Cell melanin content vs. molar concentration of 8-methoxypsoralen (MOP) and trimethylpsoralen (TMP). Bars indicate standard deviations.

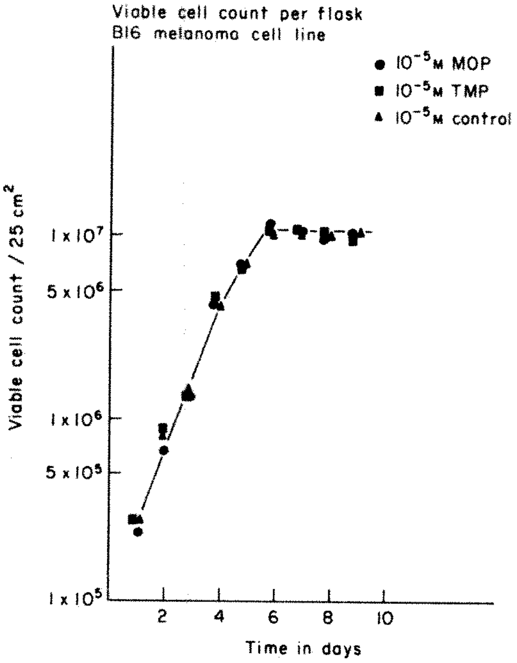

FIGURE 4. Growth curve of control B16 melanoma cells and cells treated with 10^{-5} M 8-methoxypsoralen (MOP) and trimethylpsoralen (TMP).

Electron microscopic examinations were performed on cells treated with 10^{-5} M MOP, and 0·1% DMSO (as control). Increased numbers of melanosomes were observed in those cells treated with TMP (Fig. 1a) as compared with control (Fig. 1c) and MOP-treated cells (Fig. 1b).

Determination of tyrosinase activity revealed a highly significant elevation of activity in those cells treated with 10^{-5} M TMP (Fig. 2), (Student's unpaired t-test, $P = 0{\cdot}0002$). Cells treated with lower concentrations of TMP were not significantly different from controls. At all concentrations of MOP the tyrosinase activity was not significantly different from the controls. Comparison of tyrosinase activity between cells treated with MOP and TMP showed a significant difference at 10^{-5} M ($P = 0{\cdot}0002$) but not at 10^{-6} M or 10^{-7} M concentrations ($P = 0{\cdot}28$ and $P = 0{\cdot}19$, respectively)

Determination of cell melanin content revealed a highly significant increase in those cells treated with 10^{-5} M TMP (Fig. 3) (Student's t-test, $P < 0{\cdot}00001$). Cells treated with lower concentrations of TMP were not significantly different from controls. At all concentrations of MOP, cell melanin content was not significantly different from controls. If one compares values for melanin content between MOP and TMP within each concentration, one finds significant differences at the high concentration (10^{-5} M MOP/TMP, $P = 0{\cdot}00003$) and no significant differences at lower concentrations.

Standard growth curves using control cells and cells treated with 10^{-5} M MOP and 10^{-5} M TMP were run in parallel. The curves were identical with saturation density at about 1×10^{-7} cells per flask (Fig. 4). Calculated doubling time for control cells was 28·2 h, for MOP-treated cells it was 26·7 h and for TMP-treated cells it was 28·2 h.

DISCUSSION

Stimulation of pigmentation was demonstrated in murine melanoma cells at high concentrations of TMP in the absence of UV-A. In addition, no stimulation of pigmentation was demonstrated with any concentration of MOP in the absence of UV-A in this experimental setting. Previous work (Carter *et al.*, 1974, 1979) showed no stimulation of pigment formation in the absence of UV-A with either TMP or MOP, but there are several differences between the experimental design of Carter and that of the present investigation. Carter used a short-term assay of 24 and 48 h, while in this study all assays were performed at the end of 2 weeks in culture. The reason for choosing this time is that this is when observable pigmentation of the cell pellet occurred. Kreider *et al.* (1975) have also used a 13-day culture period for B16 melanoma cells in their pigmentation studies using theophylline as an inducer of differentiation. In their studies, there was a decrease in cell turnover as differentiation (manifested by pigment production) progressed. In our study, however, there was no alteration of cell growth by 10^{-5} M MOP or TMP (Fig. 4) as evidenced by almost identical doubling times. In addition, viability was always greater than 95% as determined by the dye exclusion test after centrifugation. Therefore, the pigment production induced by TMP cannot be ascribed to cellular injury or to differentiation as a result of decreased cell division rate.

The concentration of 10^{-5} M psoralens used in this investigation was within the range of those used by Carter *et al.* (1974, 1979). In one study a concentration of 10^{-3} M TMP was used while in another the highest concentration used was 2×10^{-7} M. However, in both of these studies, the incubation period was again 24–48 hours. In neither study was pigment production induced by either TMP or MOP. In the present study, however, incubation of the B16 melanoma cells with TMP did show an increase in both tyrosinase activity and melanin content, concomitant with visible pigmentation in the cell pellet at the end of 2 weeks in culture. The control culture did not show these findings nor did the cultures exposed only to MOP, making an artifact due to culture conditions unlikely. All cultures were run in parallel with the same media and serum. It is interesting that Carter *et al.* (1979) have also shown a divergence in effect between TMP and MOP; the ability to augment tyrosinase production by melanotropin was different for these two psoralens in murine melanoma cells exposed to long wave ultraviolet light.

The mechanism for stimulation of pigmentation by psoralens is unknown. The fact that different substitutions on the psoralen molecular nucleus produce agents which have different effects on the pigmentary system of cultured cells may eventually provide the answer to this question. Carter, Wolff and Schnedl (1974) and Carter *et al.* (1979) have found that TMP and MOP bind differently to the nuclear DNA of melanoma cells. In the presence of UV-A, TMP tends to form bifunctional adducts whereas MOP tends to form monofunctional adducts. In addition, the differential effect of psoralens on stimulation of pigment production may not involve DNA binding at all. Kreider *et al.* (1975) have shown that theophylline-treated B16 melanoma cells decrease proliferation and concomitantly differentiate into larger, pigment-producing cells. It is assumed that this sequence of events is mediated through the cyclic nucleotide pathway. A G-2 block in the cell cycle could result—an effect operative in psoralen-treated B16 melanoma cells. Clinically, Parrish & Fitzpatrick (1976) have demonstrated that the combination of TMP and UV-A will produce repigmentation with less of an erythema response than the combination of MOP and UVA.

Therefore, we have shown that TMP but not MOP or control media can cause pigment production in B16 melanoma cells in the absence of long wave ultraviolet light. However, it is necessary to use higher than usual exposure times in order to observe these results. The effect might be mediated via DNA binding or non-DNA binding mechanisms, but no determination of this was attempted in the present study. One may speculate that under appropriate conditions of concentration and time, TMP might cause repigmentation in the clinical setting without the use of long wave ultraviolet light.

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In vitro growth rates of epidermal cells derived from the skin of psoriatic patients and non-psoriatic controls

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SUMMARY

The growth of epidermal cells derived from clinically normal skin of psoriatic patients and controls has been studied in culture. Growth rates were measured in secondary cultures established with 5×10^4 cells/35 mm plate without feeder layers by determining the plating efficiency, and cell yield and surface area of growth at the end of a 14-day culture period. There was no significant correlation of plating efficiency, cell yield/colony or surface area/colony with the sex or age of the donor in either the normal or psoriatic groups.

The morphological development of normal and psoriatic epidermal cell cultures was similar. Comparisons of plating efficiency, cell yield/colony and surface area/colony for the normal and psoriatic groups revealed no significant difference.

The basis of the epidermal hyperplasia which is a characteristic of psoriasis has been the subject of many studies and there is now general agreement that the cellular DNA synthetic activity and the mitotic index of cells in the involved epidermis are increased (Duffill, Wright & Shuster, 1976). Psoriasis appears to have a genetic component and several studies have related this to abnormal HLA frequencies (Gunn *et al.*, 1979).

Although the clinical manifestations appear mainly in the epidermis, the direct cause of the hyperplasia remains obscure, as epidermal cell behaviour can be influenced by both dermal and systemic factors (Wessels, 1962). Cell culture provides a means of studying epidermal cells in isolation from influences which exist *in vivo*. Chopra & Flaxman (1974) using explants measured the cell cycle time for epidermal cells derived from skin of normal subjects and from involved skin of psoriatic patients but found no significant difference. Moreover, in [^{3}H]thymidine

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incorporation studies no difference was seen in the continuous labelling indices of cells from these two sources.

In contrast, Harper, Rispler & Urbanek (1978) reported that whilst the [^{3}H]thymidine 'flash' labelling and continuous labelling indices were similar for psoriatic epidermal cells grown from explants of involved and non-involved skin, the values were considerably higher than those from cultures of normal skin. Recently Baden, Kubilus & MacDonald (1981) have examined the growth behaviour of normal and psoriatic cells cultured with human fibroblast and mouse fibroblast feeder layers but found no obvious differences. However, the extent to which cells of the feeder layer influence epidermal cell growth is unknown.

In this study we report the results of experiments to measure the growth rate of epidermal cells from the uninvolved skin of psoriatic patients and the skin of control subjects, using small inocula without feeder layers in order to examine the possibility of a genetically controlled effect on the growth of isolated epidermal cells derived from psoriatic patients.

MATERIALS AND METHODS

Cultures of epidermal cells were established from single 4 mm punch biopsies obtained from uninvolved areas of skin from the lower back of severely affected psoriatic patients of both sexes whose ages ranged from 19 to 70 years. None of the patients were on systemic therapy for psoriasis and topical therapy was confined to the plaque areas. Skin for control cultures was obtained from normal volunteers, from mastectomy specimens and from cadavers at post-mortem. The sex and age distributions (19–65 years) of these subjects were similar to that of the psoriatic patients.

The dermis on the skin biopsies was trimmed away as much as possible and the remaining tissue washed vigorously in 0·02% EDTA in phosphate-buffered saline (PBS) and cut into very small explants. These were transferred in a couple of drops of growth medium to 35 mm culture dishes containing mitomycin-treated 3T3 mouse fibroblast feeder layers prepared as described previously (West, Johansen & Faed, 1982) and incubated for 30–45 minutes at 37°C to promote explant attachment. Two ml of medium was then added to each dish. The medium consisted of F10 containing 2 mmol/l glutamine, 100 μg/ml kanamycin and 100 iu/ml benzyl penicillin, together with 20% fetal calf serum (FCS) 5 μg/ml insulin and 0·4 μg/ml hydrocortisone. The dishes were initially incubated undisturbed for 5 days and the medium then changed three times per week.

Cultures were harvested 12–17 days after initiation. The feeder layers were removed by washing with 0·02% EDTA in PBS after which the cultures were incubated for about 30 min in F10 containing 1·5 mg/ml dithioerythritol and then 0·01% trypsin and 0·02% EDTA in PBS for 10 min. Dissociated cells were suspended in medium, filtered through a 70 μm mesh nylon gauze and transferred to freezing medium (58% F10, 30% FCS and 12% dimethyl sulphoxide). Cells were stored in liquid nitrogen until required.

For the growth rate studies these cells were set up without feeder layers at 5 × 10^4 cells/35 mm dish. The medium for these cultures was as described above except that 10^{-10} mol/l cholera toxin was added. The medium was changed after 5–7 days and thereafter three times per week.

Colonies were counted either after fixation of the cultures and staining with 1% rhodanile blue or from photographs of living cultures using dark ground illumination. Plating efficiencies were determined from the number of colonies over 0·28 mm in their longest diameter after 7 days of culture. Photographs of cultures were printed at 3·55 × magnification and only colonies of diameter 1 mm or more were counted.

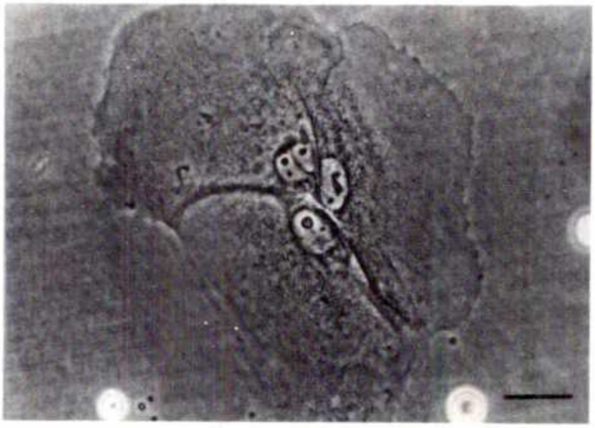

FIGURE 1. A small colony of epidermal cells consisting of three large, flattened cells with no small cells present at 7 days of culture. Bar = 30 μm.

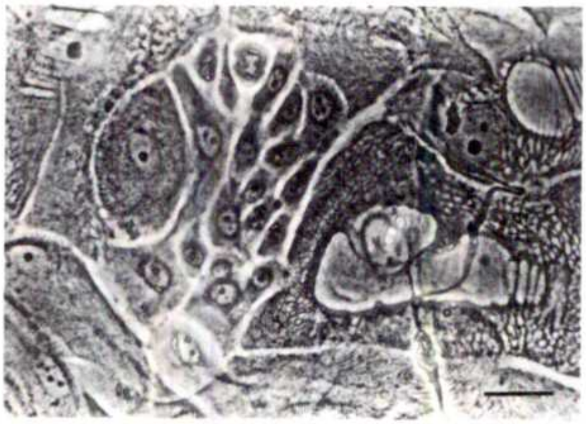

FIGURE 2. An area of small cells in a colony surrounded by large, flattened cells at 14 days of culture. Bar = 30 μm.

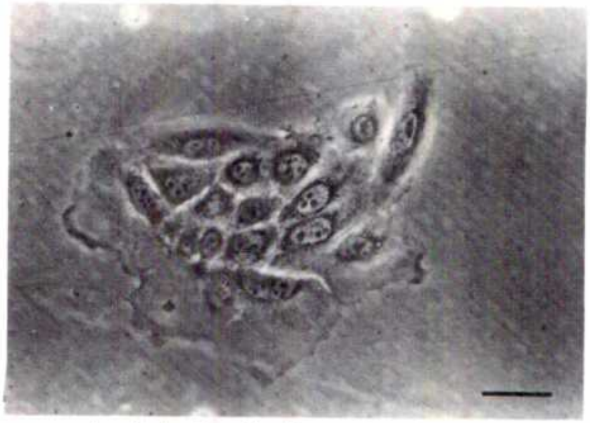

FIGURE 3. A colony composed entirely of small cells at 6 days of culture. Notice the size of these cells compared with those in Fig. 1. Bar = 30 μm.

After 14 days the cultures were photographed using dark ground illumination and the surface area of growth determined by a point counting method. A grid with a total of 434 points in sqaure lattice array arranged in a circle of equal diameter to the photograph of the culture dish surface was used for the analysis. Points on the growth were counted for three to five random orientations of the grid and the mean taken to determine the growth surface area.

Immediately after the cultures were photographed, they were harvested to determine cell yields. The dissociated cells were suspended and washed in medium and counted in an improved Neubauer haemocytometer.

RESULTS

Successful cultures for the growth rate measurements were established from six normal and seven psoriatic samples. During the first few days in culture, small colonies of mainly large flattened cells formed, many of which differentiated (Fig. 1). Some colonies showed no further development. However, in other colonies, after the first few days and thereafter, discrete areas of small, rapidly dividing cells appeared and these became the main growth areas (Fig. 2). Very occasionally a few of the initial colonies were composed entirely of small cells which tended to grow rapidly (Fig. 3). Different development of individual colonies resulted in a great variation in colony size on each culture dish. By the end of the culture period small areas of multilayering were often found in the larger colonies. Some differentiation had occurred in the upper layer of multilayered colonies, the cells appearing large and flattened with thickened cell borders. The morphology of the cells from normal subjects and psoriatic patients was similar and there was no noticeable difference in culture development of cells from the two sources.

TABLE 1. Plating efficiencies and mean colony sizes for normal and psoriatic epidermal cell cultures after 14 days growth

Culture Type* Age (years)	Sex	Plating efficiency	Cell yield/ colony	% Surface area/ colony $\times 10^3$
Normal				
19	M	0·51 ± 0·02	140 ± 23	26·2 ± 1·8
28	M	0·93 ± 0·04	395 ± 37	95 ± 2·5
46	F	0·82 ± 0·05	76 ± 14	20·3 ± 3
54	M	0·73 ± 0·05	23 ± 7	6·9 ± 0·9
60	F	0·81 ± 0·05	40 ± 7	14·9 ± 2·1
65	F	0·92 ± 0·03	17 ± 2	6·2 ± 1·2
Overall mean	± sd	0·79 ± 0·15	115 ± 144	28·3 ± 33·6
Psoriatic				
19	F	0·89 ± 0·12	53 ± 20	9·1 ± 1·4
33	F	0·82 ± 0·05	110 ± 12	21·7 ± 2
35	M	1·12 ± 0·03	511 ± 41	85·9 ± 11·1
51	M	0·85 ± 0·03	25 ± 1	6·4 ± 1·1
53	F	0·5 ± 0·03	216 ± 14	41·5 ± 3·6
58	F	1·03 ± 0·02	58 ± 7	31·1 ± 2·3
70	F	0·72 ± 0·03	92 ± 17	21 ± 1·1
Overall mean	± sd	0·85 ± 0·2	155 ± 168	31·8 ± 27·6

* Data for each sample given as mean ± standard error.

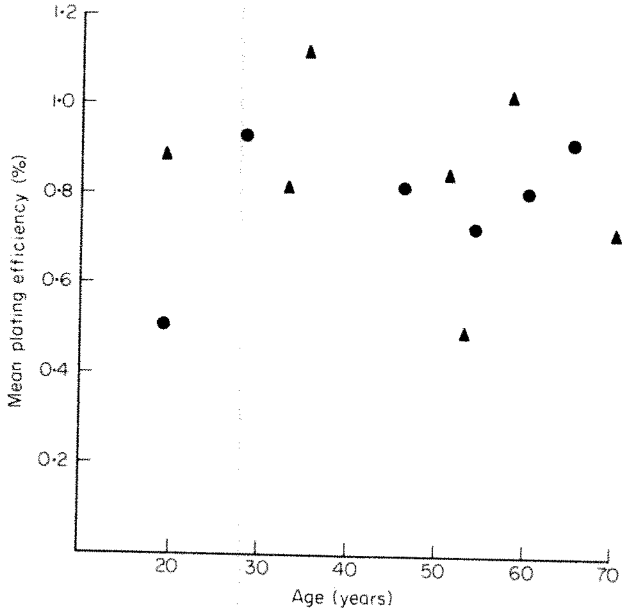

FIGURE 4. The mean plating efficiency of epidermal cells against age of donors of skin samples. The variation in plating efficiency for both normal and psoriatic epidermal cell cultures is not large. (●) Control cultures; (▲) Psoriatic epidermal cell cultures.

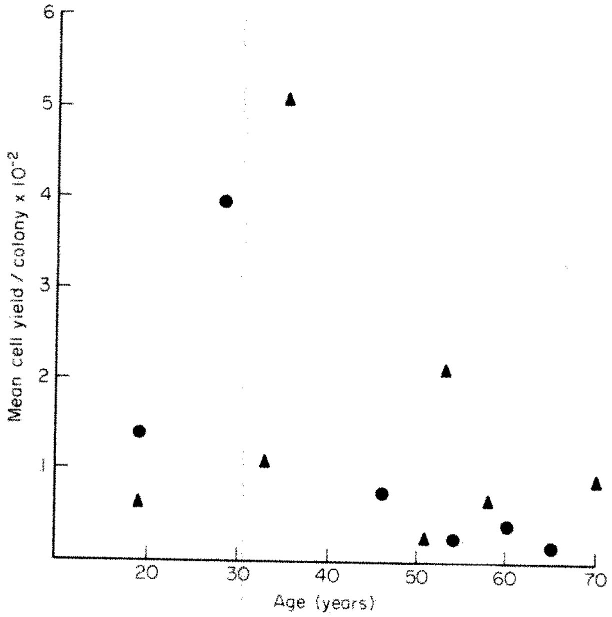

FIGURE 5. Mean cell yield/colony against age of skin donor. There is a large inter-sample variation in the mean colony size for both the normal (●) and psoriatic (▲) epidermal cell cultures.

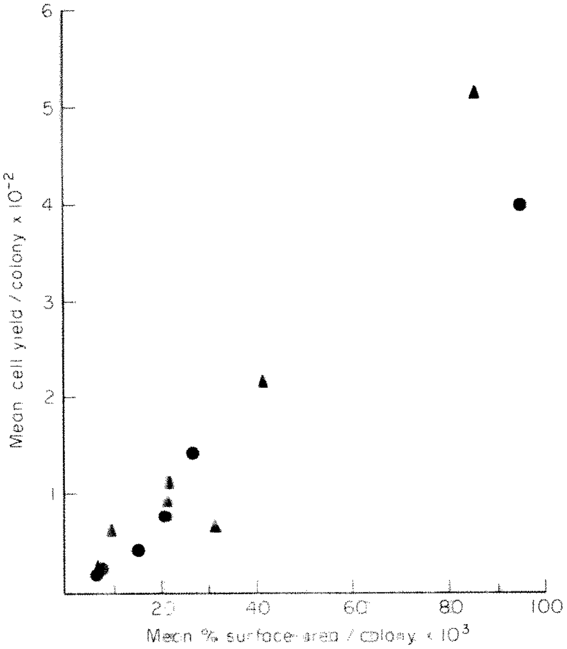

FIGURE 6. Mean cell yield/colony against mean % surface area/colony. The graph shows similar ratios of these two measurements for the control (●) and psoriatic (▲) epidermal cell cultures.

The quantitative results are given in Table . There were no sex-associated differences in plating efficiency, cell yield and surface area per colony for either the normal or psoriatic groups and the results have therefore been analysed regardless of sex.

Variation in plating efficiency is not great (Fig. 4), but the inter-sample variation in cell growth as measured by either cell yield/colony (Fig. 5) or surface area/colony (Table 1) is large. An analysis of the variance of plating efficiency cell yield/colony and surface area/colony with age as a covariate using a generalized linear interactive modelling (GLIM) programme package revealed no significant correlation of these measurements with age.

Comparison of these measurements for the psoriatic and control cultures revealed no significant difference. With the variation of the samples in both of these groups, and assuming no age effect, the difference in mean plating efficiency between the normal and psoriatic groups would have to be greater than 0·23% to be significant at the 5% level. Similarly, the differences in mean cell yield/colony and surface area/colony for both groups would have to be greater than 200 cells and 0·038% respectively to be significant.

The ratio of cell yield/colony to surface area of growth/colony should give an indication of the extent of mutilayering in the cultures. However, a comparison of these ratios for the psoriatic cell cultures and the controls revealed no significant difference in this respect either (Fig. 6).

DISCUSSION

The investigation was designed to study the growth behaviour of epidermal cells from normal subjects and psoriatic patients in isolation from any other cell type so that a genetically determined effect intrinsic to the epidermal cell may become apparent. No morphological or quantitative differences between cultures from the two sources have been found, but the variation was such that only gross differences would be detected. The variation does not appear to be due to sex or age of the donor of the skin sample.

Both cell yield and surface area were measured as criteria of growth since differences in one aspect of differentiation, multilayering, would be reflected in the cell yield: surface area ratio. The more extensive the multilayering, the higher the ratio of these measurements. However, direct observation of the normal and psoriatic cultures had suggested little difference in multilayering and this is supported by the cell yield/surface area (Fig. 6) which is similar for both groups.

Although no differences have been detected between the normal and psoriatic cultures, only the early culture period has been studied in which there was neither extensive differentiation nor the formation of a continuous epithelium. The results do not exclude the possibility of a difference in later cultures where both these features would be present and may differentially affect the size of the proliferating cell pool in cultures of cells from both sources.

The psoriatic cells used in this study were derived from non-involved areas of the skin but there is no evidence to suggest that epidermal cells from such sites are genetically distinct from those in active psoriatic lesions. Harper *et al.* (1978) found no difference in [^{3}H]thymidine labelling indices of cultured psoriatic cells from involved and non-involved skin, although both were reported to be above control values. Chopra & Flaxman (1974) found that the cell cycle times and growth fractions of cells cultured from involved psoriatic skin and from the skin of normal subjects were similar, and Baden *et al.* (1981) using fibroblast feeder layers found no difference in growth of cells cultured from similar sources. Our results using cells cultured in isolation would seem to show that there is no difference in growth rate between cells derived from the uninvolved skin of psoriatic patients and cells from normal people.

We have attempted to examine the growth characteristics of epidermal cells separated from the fibroblasts of the dermis with which they are associated *in vivo* but it is conceivable that the psoriatic plaque is initiated by abnormal stimulation from the underlying dermis. Fibroblasts derived from normal and non-involved psoriatic dermis when used as feeder layers support epidermal cell growth to a similar extent but both are far less efficient in this respect than 3T3 cells (unpublished observations). Baden *et al.* (1981) also report similar results for fibroblasts isolated from the psoriatic plaque.

Under the conditions of this study, no detectable abnormality was found in the psoriatic epidermal cells. It remains to be seen if the growth conditions of the early proliferative phase in culture are appropriate for manifesting inherent differences in epithelial cell growth and differentiation.

ACKNOWLEDGMENTS

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Adenylate cyclase activity during growth and maturation of keratinocytes: comparison of two methods of study

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SUMMARY

The adenylate cyclase activity of primary cultures of guinea-pig ear keratinocytes was studied at different times by challenge with various agonists after labelling of ATP by exposure of the cells to [^{3}H]-adenine. The [^{3}H]-cAMP (cyclic adenosine-3′,5-monophosphate) formed was assayed by scintillation counting. In other experiments, cells were exposed to the equivalent amount of unlabelled adenine, challenged and cAMP was then assayed by radioimmunoassay. These absolute amounts were compared to [^{3}H]-cAMP levels expressed as percentage conversion of [^{3}H]-ATP.

Comparison of the two methods suggested that the use of the prelabelling method would result in underestimation of cAMP formed. It was found that the response of the enzyme to different agonists changed with the length of time of the cells in culture.

Cyclic AMP is usually, if not always, associated with the commitment of cells to differentiate or to express differentiative properties, or both (Friedman, 1976). However, the demonstration of these functions in epidermal tissue has not been accomplished. Cytochemical detection of adenylate cyclase (ACase) activity has shown that this enzyme is localized in the basal stratum and in several suprabasal layers of the epidermis, and can be stimulated by various agonists (Mahrle & Orfanos, 1975; Sondergaard, Wadskov & Kobayasi, 1977). This suggests that cAMP may function in and beyond the proliferative compartment. Examining sequential slices of bovine snout epidermis, Gommans *et al.* (1979) found that cAMP was concentrated towards the basal layer. On the other hand, microdissection and assay of human epidermis revealed that cAMP was distributed evenly throughout the tissue (Aoyagi, Kamigaki & Miura, 1980). On the basis of available evidence, the localization of cAMP is uncertain.

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Keratinocyte cultures represent epidermal model systems that are potentially useful in the study of mechanisms involved in aspects of epidermal differentiation. In general, after plating out, keratinocytes pass through a lag phase, a growth phase, and a prolonged plateau phase characterized by stratification, desquamation of keratinizing cells, diminished mitotic activity and the appearance of markers of differentiation (Liu & Karasek, 1978). It is possible that cyclic nucleotides participate in biochemical events during these stages, and their presence can be estimated either directly or by assay of the activity of their formative enzymes. In this study, ACase is evaluated by its response to several agonists, but principally to isoproterenol, leading to the formation of unlabelled cAMP (or labelled cAMP, if [^{3}H]-ATP is used). The method using prelabelled ATP is compared to radioimmunoassay for quantification of cAMP.

METHODS

Cell culture

Keratinocytes were harvested from guinea-pig ears (Hartley albino, male, 300 g) and cultured as described by Mahrle, Wilkinson & Farber (1981).

Adenylate cyclase assay

At intervals of 2 or 3 days, dishes of cells were selected at random in triplicate, the medium was aspirated, and [^{3}H]-adenine (5 μCi in 1·5 ml of serum-free medium) was added to each. Three other dishes were treated with the equivalent amount of *cold* adenine (45 ng, based on specific activity of 15·0 Ci/mmol) in 1·5 ml of medium. After 2 h at 37°C, the media were aspirated from all dishes, the cell layers were rinsed with fresh medium at 37°C, and 2·0 ml Dulbecco phosphate-buffered saline at 37°C was added, containing 1·0 mmol/l 3-isobutyl-1-methylxanthine (IBMX), Hepes (15·0 mmol/l) and (−)-isoproterenol (10 μmol/l). In some experiments using [^{3}H]-adenine, additional agonists were tested: histamine (100 μmol/l), adenosine (100 μmol/l) or PGE$_1$(10 μmol/l). Control dishes received buffer with IBMX and Hepes only. After 15 min at 37°C, the media were removed and ice-cold TCA (5%) was added to the cell layers. Precipitated material was collected by scraping, brief homogenization and centrifugation (10,000 rpm/20 min). After extraction with water-saturated ether to remove TCA, the supernatants were lyophilized and the residues dissolved in water (0·8 ml) containing [^{14}C]-cAMP (about 4000 dpm).

In the case of samples containing prelabelled ATP, aliquots (50 μl) were taken and dissolved in aqueous scintillation counting fluid (ACS; 12·0 ml) for radioactivity counting (^{3}H channel; 43% efficiency). These cpm × 16 gave the total ^{3}H (ATP + cAMP). Cyclic AMP in the remainder was purified as described by Salomon, Londos & Rodbell (1974) with the following modification: effluents from alumina columns were lyophilized in scintillation vials and the residues redissolved in water (0·5 ml) and ACS (12·0 ml) before 2-channel counting (^{3}H at 43% and ^{14}C at 86% efficiency). [^{3}H]-cAMP values were corrected for recovery and expressed as per cent of total ^{3}H.

In the case of samples containing cold ATP, cAMP was purified by passage of the 0·8 ml solutions through the AG 50W-X4 resin columns (Salomon, *et al.*, 1974), followed by lyophilization of the eluate and dissolution of the residue in the appropriate buffer for RIA assay as before (Wilkinson, Liu & Orenberg, 1981). Aliquots of this final solution were counted in ACS on the ^{14}C channel to estimate recovery.

Radiopurity of [^{3}H]-ATP

Aliquots of TCA-free supernatants were spotted on PEI-cellulose plates which were developed sequentially in LiCl (0·3 mol/l; 1·0 min), LiCl (1·0 mol/l; 12·0 min) and LiCl (1·6 mol/l; 47·0 min). Samples of adenosine, AMP, ADP and ATP were run alongside. The wet plates were inspected in 254 nm light and the location of these compounds marked. Bands were ruled on the dry plates, and were scraped off into vials for scintillation counting after Protosol digestion and addition of ACS.

DNA assay

DNA was determined using ethidium bromide on aliquots of solutions of pelleted material in NaOH (Halprin *et al*, 1979).

Sources of materials

Sigma Chemical Co.: Hepes, adenine, (−)-isoproterenol bitartrate, L-histamine HCL, IBMX, PGE$_1$, ethidium bromide, alumina. Gibco Laboratories: cell culture supplies. Schering Corporation: gentamicin (Garamycin®). E.R. Squibb & Sons, Inc.: amphotericin B (Fungizone®). New England Nuclear: 2-[^{3}H]adenine (spec. act. 15 Ci/mmole), 8-[^{14}C]cAMP (48 mCi/mmole), methyl-[^{3}H]thymidine (6·7 Ci/mmole) and Protosol. Amersham Corporation: ACS. Collaborative Research Inc.: RIA materials, except cAMP antiserum (gift of Dr Gary Brooker).

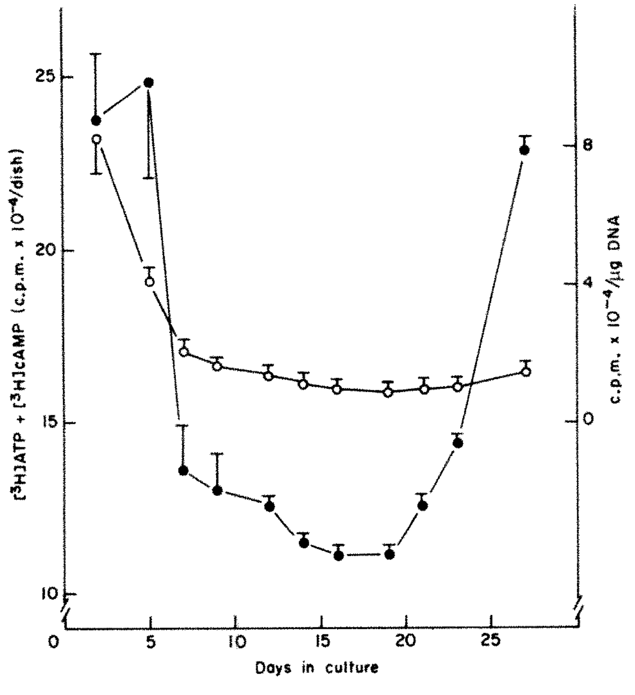

FIGURE 1. Incorporation of [^{3}H]adenine into [^{3}H]ATP (including [^{3}H]cAMP formed by challenge with (−)-isoproterenol) at various times during the first 27 days of the culture period. Values are means ± s.e. from three determinations and are expressed as radioactivity per dish (●) or per µg DNA (○).

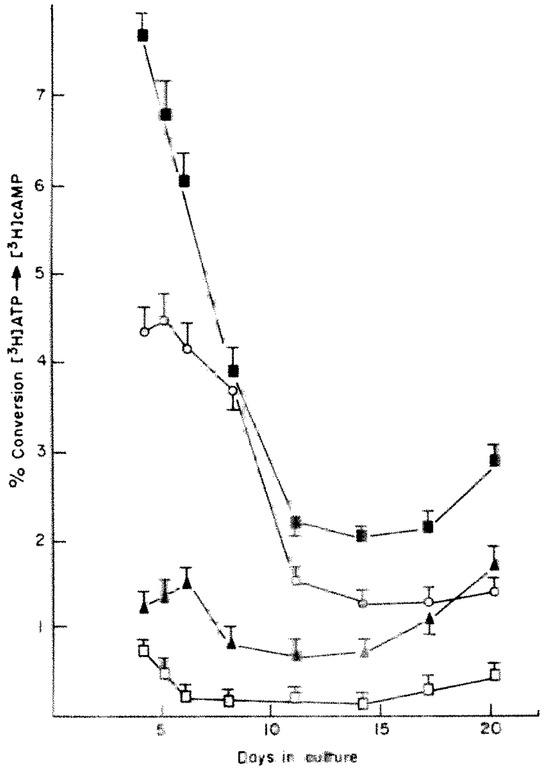

FIGURE 2. Per cent conversion of prelabelled ATP to cAMP in keratinocytes at various times during the first 20 days of the culture period, on challenge with L-histamine (100 μM, ■), (−)-isoproterenol (10 μM, ○), PGE$_1$ (10 μM, ▲) or adenosine (100 μM, □). Values are means ± s.e. from three determinations. These patterns have been verified in two separate experiments.

RESULTS

Labelling of ATP from [^{3}H]-adenine

Keratinocytes incorporated [^{3}H]-adenine into ATP in a linear fashion over a period of 2 h. Separation of adenine nucleotides on PEI-cellulose thin layer plates showed that over 80% of the label was consistently associated with ATP. Maximal amounts of [^{3}H]-adenine were incorporated during the first 7 days of the culture period which is the period of most rapid growth (Fig. 1). This was followed by a sharp decline; when expressed in terms of DNA, [^{3}H] ATP levels remained fairly constant (Fig. 1).

ACase activity

Prelabelled ATP method. The percentage conversion of [^{3}H]ATP to [^{3}H]cAMP on challenge with various agonists is shown in Fig. 2, and with isoproterenol alone (in more detail) in Fig. 3. Clearly, the relative sensitivity of cultured keratinocytes to different compounds varied during the *in vitro* lifespan of the cells. With time, the response to PGE$_1$ became increasingly significant and equalled the effectiveness of catecholamines (Fig. 2). In all cases the sensitivity tended to

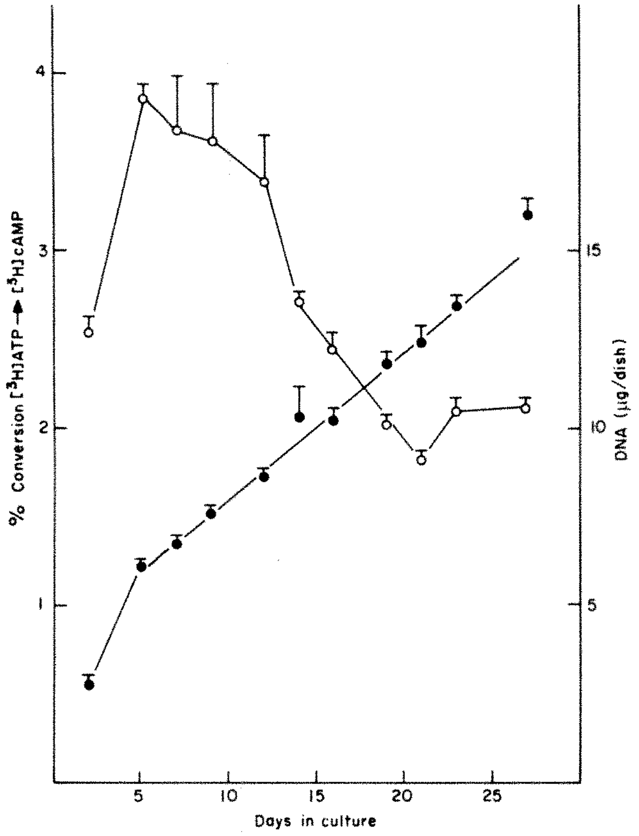

FIGURE 3. Per cent conversion of prelabelled ATP to cAMP in keratinocytes at various times during the first 27 days of the culture period, on challenge with (−)-isoproterenol (10 μM). Values (○) are means ± s.e. from three determinations. The growth curve is also shown; values (●) of DNA are means ± s.e. from six determinations.

increase around day 20. Maximal responses were observed during days 5–12; the sensitivity curve showed a shoulder at day 12, then fell off sharply (Fig. 3). This period of maximal sensitivity did not clearly correspond to the period of greatest growth.

RIA method. In the same experiment, from which the data in Fig. 3 are obtained, the cAMP generated on isoproterenol challenge of cells pre-incubated with cold adenine was measured. A similar time-course curve was obtained (Fig. 4). Both methods revealed maximal sensitivity on day 5, a shoulder in ACase response at about day 12, and an increase in activity around day 20.

Comparison of ATP prelabelling and RIA methods:
Using the % conversion (Fig. 3), and the data for cAMP levels per dish (Fig. 4), the two methods were compared by expressing the latter in terms of a 1% conversion of [^{3}H]ATP. The results are shown in Fig. 5. The amount of cAMP corresponding to a 1% conversion on challenge with isoproterenol increased from 80 pmol on day 2 to over 300 pmol on day 20.

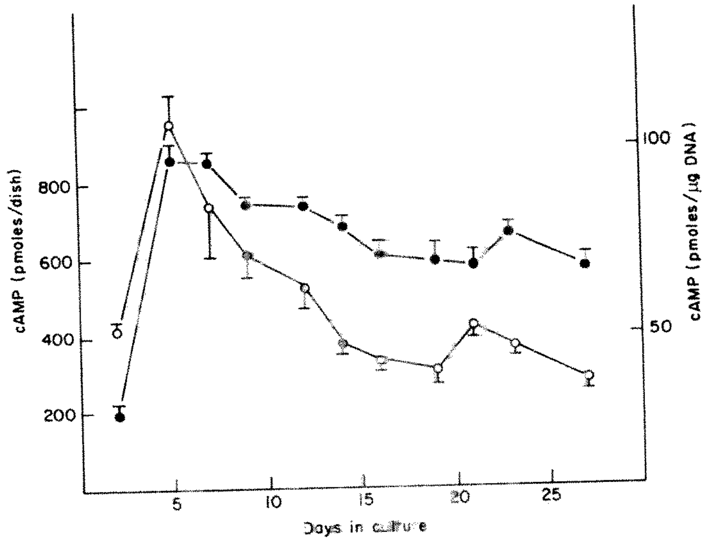

FIGURE 4. Cyclic AMP levels in cultured keratinocytes pre-incubated with the amount of unlabelled adenine equivalent to 5 µCi of [^{3}H]adenine, and challenged with (−)-isoproterenol (10 µM). Values are means ± s.e. from three dishes and are expressed in terms of cAMP per dish (●) or per µg DNA (○).

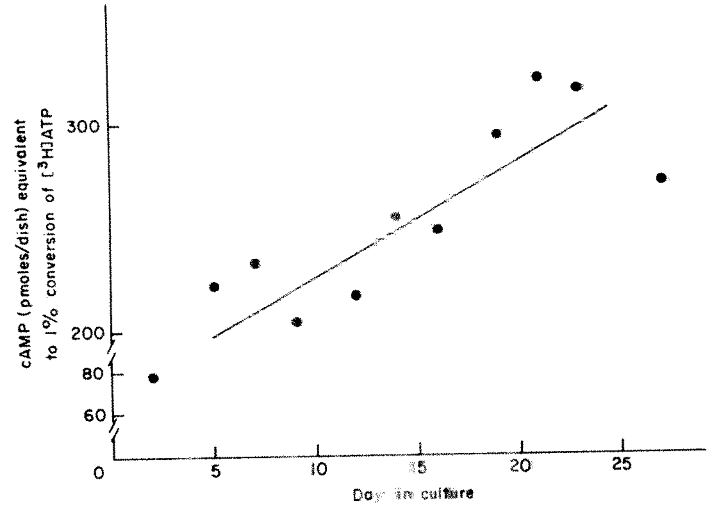

FIGURE 5. The amounts of cAMP equivalent to 1% conversion of [^{3}H]ATP at various times during culture.

DISCUSSION

Many cells and tissues incorporate [^{3}H]adenine into [^{3}H]ATP (Murray, Elliott & Atkinson, 1970), and the conversion of [^{3}H]ATP to [^{3}H]cAMP has been the basis for a number of studies of adenylate cyclase (Kebabian, Kuo & Greengard, 1972). In dermatological research, the two reactions have been utilized in, for instance, a study of healthy and psoriatic epidermis; the latter shows reduced ability to form [^{3}H]ATP or [^{3}H]cAMP (Wright *et al.*, 1973). The method is attractive for its convenience, economy and precision, and for the possibility of further adaptation. For instance, the fate of [^{3}H]cAMP (degradation by phosphodiesterase, migration from the cell, etc.) is easily followed. The main drawback is the impossibility of ascertaining that the endogenous ATP pool is labelled in a representative manner. Receptors for different agonists of adenylate cyclase may occur in clusters in different locations on the cell surface and the enzyme may have access to ATP pools that are not necessarily labelled identically. There is no evidence that this is the case in keratinocytes, although the location of other membrane-associated enzymes such as ATPase has been demonstrated histochemically to occur on the side of the basal layer facing the dermis (Mahrle & Orfanos, 1975).

Cyclic AMP was also measured after pre-incubation of cells with the equivalent amount of cold adenine, and challenge by isoproterenol. In other experiments it was found that this exposure to adenine did not increase the yield of cAMP (Wilkinson & Orenberg, unpublished). Thus, [^{3}H]ATP behaves as a true tracer and has negligible effect on the size of the ATP pool. The RIA method measures basal as well as stimulated levels of cAMP, but the former are only of the order of 4 pmoles per dish (10 pmoles at day 5) and make negligible difference to the data of Fig. 4. Comparison of the response curves indicates that although the main features are reproduced by both methods (e.g., the period of maximal formation of cAMP), pre-incubation with [^{3}H]adenine for 2 h labels the ATP pool(s) in a manner that becomes increasingly inadequate with the age of the cultures. The percentage conversion of [^{3}H]ATP falls by 50% between days 5 and 20, but the amount of cAMP formed falls by 29%. The data are consistent with the existence of partially labelled or unlabelled ATP pools which become increasingly significant with the culture age.

In previous studies using passaged human keratinocytes maintained in culture for as long as 60 days, it was found that basal levels of cAMP (Wilkinson *et al.*, 1981) or adenylate cyclase activity (Wilkinson *et al.*, 1982) were highest during the period of greatest DNA synthesis and mitotic activity. Similar patterns were observed here, although the association with growth was less obvious. The linearity of the growth curve in terms of DNA suggests the presence of a mitotic subpopulation of constant size and the gradual decrease in amounts of cAMP per μg DNA can be interpreted as due to the accumulation of maturing keratinocytes that still retain their DNA, but have lower cAMP content.

Isoproterenol-sensitive ACase has been demonstrated in the basal layers and in four to five suprabasal layers in human epidermis (Sondergaard *et al.*, 1977) and in basal cells in mouse (Verma *et al.*, 1976). Glucagon-responsive activity was seen throughout human epidermis (Mahrle & Orfanos, 1975). It is possible that ACase stimulation by different agonists shows localization; in other words, the keratinocyte may display receptors for different agonists at various maturation stages. Our data (Fig. 2) suggest that prostaglandins may increase in significance in formation of cAMP at later stages of keratinization.

The increase in [^{3}H]adenine incorporation and adenylate cyclase activity which commences at days 17–20 is under investigation. It may represent an episodic increase in the proportion of cycling cells, as encountered before by observation of the mitotic index (Wilkinson *et al.*, 1981). Such periods show a transient increase in enzyme activity. Alternatively, it may presage an event related to differentiation. Recently, the calcium-calmodulin activation of transglutaminase has been demonstrated (Peterson, Zettergren & Wuepper, 1982); this enzyme is involved in cross-linking of keratin proteins. It is not known whether cAMP modulates aspects of this metabolism, but certainly cAMP and calcium are frequently associated in 'synarchic' fashion in a great many biological systems (Rasmussen, 1981). The β-adrenergic response can be induced to appear during the differentiation of many cell types (Lin & Lin, 1979). Its occurrence during or preceding the appearance of differentiated characteristics in keratinocytes would appear to be perfectly plausible.

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Diffusion barriers in skin—a new method of comparison

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SUMMARY

Using a method which allows the net barrier potential of skin to be expressed in terms of a 'penetration half-life', skin barriers to nandrolone and allyl oestrenol penetration were compared in human, rat and hamster skin. The ranking order in terms of the net barrier was: human = haired rat ⩾ hairless rat > hairless hamster. Using the same principles, the net barrier effects of the various layers in whole human skin were also compared. The dermis was only slightly less effective than the stratum corneum as a barrier to diffusion.

Over the past two decades or so, a considerable body of experimental work has established an understanding of the rates, mechanisms and routes by which chemical compounds penetrate skin. Almost all such experiments are based on an analysis due originally to Daynes (1920), which requires the skin sample to be mounted with both faces in continuous contact with the (usually aqueous) solvent. The results therefore relate to skin which is saturated with water. Since it is well known that the water content of the stratum corneum has a pronounced effect on penetration rates, this is a major disadvantage of the conventional method, and imposes severe limitations on the generality of the results obtained.

An alternative method has been described in which a thin film of material is applied to the skin surface (Foreman, Kelly & Lukowiecki, 1977). This method can be employed in such a way that both occluded and non-occluded skin may be studied. An additional advantage, discussed here in more detail, is that since a finite amount of diffusant is applied to the skin surface, the 'thin film' method allows the diffusion process to be expressed in terms of a 'penetration half-life' ($T^{D}_{\frac{1}{2}}$), being the time taken for one-half of the *diffusing* material to penetrate the skin sample. Such a measurement is more immediately useful than the diffusion constant alone, in that it reflects the net barrier effect of the sample in an easily assimilated manner. In the present case, therefore, this approach has been applied to a limited interspecies comparison of skin barriers to nandrolone and allyl oestrenol penetration, and to a comparison of the barrier effect of the various layers which make up whole human skin.

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MATERIALS AND METHODS

Skin samples

Whole human abdominal cadaver skin was prepared by removing subcutaneous fat. Samples were stored at 4°C prior to use. Layers of epidermis and dermis were obtained by placing the dermal side of whole skin against an asbestos sheet, the epidermal side against a metal sheet, and immersing the whole, clamped together, in water at 60°C for 2 min. The epidermis was then peeled away from the dermis. Sheets of stratum corneum were obtained by overnight digestion of separated epidermis in 0·2% trypsin.

Whole hairless hamster skin was obtained from animals sacrificed by ether inhalation. The fat layer was removed by careful scraping with a scalpel blade. Hamster skin was used immediately or within 24 h. Rat skin was obtained in a similar way. Skin from haired rats was clipped prior to use, which was always within 24 h of being removed from the animal.

Female hairless hamsters (WN strain) were supplied by Wrights of Essex, male hairless rats (Wistar-A.G.) were supplied by Bantin & Kingman, and male haired Wistar rats were obtained from Hacking and Churchill.

Radiolabelled diffusants

[^{14}C]nandrolone, sp. act. 40 mCi/mM, was used in solution at 10 μCi/ml. [^{3}H]allyl oestrenol, sp. act. 8·0 mCi/mM, was used at 40 μCi/ml. Diffusant was applied to the skin surface using a fine brush to ensure application as a thin film. Approximately 50 μl of solution were applied on each occasion.

Diffusion measurements

The approach used here requires that the diffusant be applied to the membrane surface as a thin film, the lower face being kept in contact with an effectively infinite sink, where the diffusant concentration is at all times zero. The apparatus and methods for meeting these conditions have been described previously (Foreman, Clanachan & Kelly, 1978). For direct measurements of diffusion in epidermis and stratum corneum, the method was slightly modified, these fragile membranes tending to rupture in the conventional apparatus. The samples of stratum corneum were placed, face up, on sheets of filter paper, and samples of epidermis were placed on polythene sheets. The diffusant was then painted on to the surface. The samples were carefully floated on to the surface of the sink solution in a suitable container, the filter paper or polythene mounts rapidly becoming separated from the skin membrane being studied. Great care was taken to ensure that the sink solution did not come into contact with the surface of the membrane. At suitable intervals the sink solution was removed and fresh solution introduced. In all cases, the diffusant in the sink solution was estimated by evaporating the sample to dryness, re-dissolving in Bray's scintillator, and counting the level of radioactivity using a Unilux II scintillation counter. The residual radioactivity remaining within the skin sample was finally estimated by digestion with Soluene 350 and counting as before. This yielded an estimate of the fraction M/M_{true} (see below). The membrane thicknesses were obtained in each case by repeated measurements over the whole surface with a micrometer gauge.

Using the above procedures, diffusion curves of the cumulative amount of diffusant penetrating each membrane as a function of time were constructed. The analysis of these curves is based on computer simulation of the ideal behaviour of a membrane under the conditions described above. The simulation procedure is described elsewhere (Foreman *et al.*, 1977), involving a membrane of length L containing particles of diffusant for which the root mean

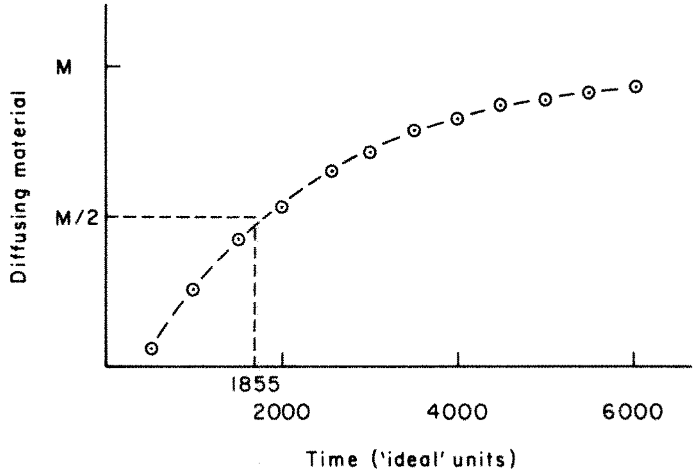

FIGURE 1. Simulated diffusion curve for penetration of material applied as a thin surface film through a plane membrane. 'Ideal units' of time = real time × S_t. $S_t = \frac{(L/50)^2}{2D}$ (Foreman *et al.*, 1977). Diffusion half-time = time taken for quantity M/2 to penetrate = 1855 $S_t = 0{\cdot}371\ L^2/D$.

square path length in unit time is L/50. The simulated diffusion curve so obtained is shown in Fig. 1. By manipulating the ideal and experimentally observed diffusion curves as described earlier (Foreman *et al.*, 1977) an estimate of the function L^2/D is obtained; D may then be calculated from a knowledge of L.

From the curve of Fig. 1, it may be seen that one-half of the diffusing material crosses the ideal membrane in a time of 1855 ideal units ($T^D_{\frac{1}{2}}$). This can be shown to be equivalent (see Fig. 1) to 0·371 L^2/D units of real time. $T^D_{\frac{1}{2}}$ may be calculated from the function of L^2/D without a knowledge of L, and in principle $T^D_{\frac{1}{2}}$ is more accurately determined by this method than D itself, since, for thin deformable membranes such as those used here, L cannot be determined with high precision. Biological variability between different samples of skin, however, tends to dilute this advantage of $T^D_{\frac{1}{2}}$ over D.

RESULTS AND DISCUSSION

Diffusion constants for nandrolone in human, rat and hamster skin are quoted in Table 1. A comparison between haired and hairless rat skin was included to provide insight into the importance of shunt diffusion in rat skin, since, although not strictly hairless, the hairless rat has fewer and narrower follicular openings to the skin surface. From the data it is evident that the diffusion constants observed (which are an 'average' of the constants appropriate to the various layers comprising 'whole skin') differ appreciably. Because of the differences in skin thickness also observed, however, it is not at all easy to decide which skin type provides the greatest net barrier. In such a case, the measurement $T^D_{\frac{1}{2}}$ is of particular value in that, with one qualification (see below), it *is* representative of the net skin barrier.

Also shown in the Table is the 'diffusing fraction' here denoted M/M_{true}. In earlier studies (Foreman *et al.*, 1978) it was found that a certain fraction of the applied material appeared to be immobilized, and took no further part in the diffusion process. The time course for this

TABLE 1. Diffusion of nandrolone in various species, non-occluded. $T = 22 \pm 2$ °C

Species	Thickness, L (cm)	Diffusion constant, D (10^{-8} cm^2 s^{-1})	Diffusing fraction M/M_{true}	Diffusion half-time $T^D_{\frac{1}{2}}$ (h)
Human	0·123 ± 0·005	3·2 ± 0·[illegible]	0·35 ± 0·01	61 ± 7
Hairless hamster	0·013 ± 0·005	0·10 ± 0·[illegible]	0·46 ± 0·01	10 ± 2
Haired rat	0·083 ± 0·005	1·2 ± 0·[illegible]	0·43 ± 0·02	60 ± 7
Hairless rat	0·107 ± 0·005	2·9 ± 0·[illegible]	0·40 ± 0·05	42 ± 6

'binding' process seems to be fast in relation to diffusion, in that, whilst occlusion increases M/M_{true} (for nandrolone) from approximately 0·[illegible] to 0·8, the diffusion constant is affected only marginally. If the two processes operated over a similar time span, such a change in M/M_{true} would most probably have markedly affected the measured diffusion constant. From the effect of occlusion on the observed value of M/M_{true}, it also seems probable that this behaviour is related to the reservoir effect reported from human vasoconstrictor studies of corticosteroids. However, this phenomenon adds an additional complication, and it must be remembered that $T^D_{\frac{1}{2}}$ is the penetration time for one-half of the *diffusing* fraction, and not the total amount applied. To obtain a complete picture of the net diffusion process therefore, both $T^D_{\frac{1}{2}}$ and M/M_{true} should be considered alongside D.

From the values of $T^D_{\frac{1}{2}}$ quoted, it can now be seen fairly readily that the rank order of skin types in terms of the net barrier to nandrolone is: haired rat = human > hairless rat > hairless hamster. These results are interesting in that whilst the skins of the haired and hairless rats have rather different diffusion constants, the net penetration barriers exerted by the two skin types are very similar, probably as a consequence of the greater thickness of the hairless rat skin. Further, if shunt diffusion were important for nandrolone, the hairless rat skin should present the greater barrier, particularly in view of its greater thickness. The opposite is true however; the haired rat skin presents slightly the greater barrier. Unless, therefore, there is a more fundamental difference between the two skin types, these results again suggest that shunt diffusion is not important for nandrolone.

The situation is similar for allyl oestrenol (Table 2). The order now appears to be human = haired rat = hairless rat > hairless hamster, although the wide variability observed makes the rank order a little less certain.

The slow diffusion rates observed in this case add a further complication. Although, in our experience, $T^D_{\frac{1}{2}}$ can be estimated accurately by studying relatively small portions of the

TABLE 2. Diffusion of allyl oestrenol in various species, non-occluded. $T = 22 \pm 2$ °C

Species	Thickness, L (cm)	Diffusion constant, D (10^{-10} cm^2 s^{-1})	Penetration fraction M/M_{true}	Diffusion half-time $T^D_{\frac{1}{2}}$ (h)
Human	0·094 ± 0·010	28·9 ± 7·8	0·12	356 ± 60
Hairless hamster	0·016 ± 0·005	1·49 ± 0·3[illegible]	0·49	179 ± 42
Haired rat	0·09 ± 0·01	32·5 ± 5·4	0·14	282 ± 74
Hairless rat	0·10 ± 0·01	36·1 ± 0·9	0·18	307 ± 12

TABLE 3. Diffusion of nandrolone in human skin layers, non-occluded. T = 22 ± 2°C

Layer	Thickness, L (cm)	Diffusion constant, D (10^{-8} cm^2 s^{-1})	Penetration fraction M/M_{true}	Diffusion half-life $T^D_{\frac{1}{2}}$ (h)
Whole skin	$0\cdot123 \pm 0\cdot005$	$3\cdot2 \pm 0\cdot5 \times 10^{-8}$	$0\cdot35 \pm 0\cdot10$	61 ± 7
Stratum corneum	$\sim 0\cdot001$	$3\cdot2 \pm 1\cdot4 \times 10^{-12}$	—*	25 ± 2
Epidermis	$\sim 0\cdot002$	$16 \pm 7 \times 10^{-12}$	—	38 ± 12
Dermis	$0\cdot124 \pm 0\cdot011$	$8\cdot9 \pm 0\cdot7 \times 10^{-8}$	$0\cdot88 \pm 0\cdot05$	18 ± 2

* Under the method employed for studies on stratum corneum and epidermis, it was not possible to obtain estimates of M/M_{true}.

experimental curve, to estimate M/M_{true} requires an extrapolation of the curve to its limit. How accurate this extrapolation is depends on how much of the experimental curve has in fact been studied; as a guideline, $2T^D_{\frac{1}{2}}$ seems to be the minimum. Nandrolone presented few problems in this respect. For allyl oestrenol, however, skin samples should at least be measured over 25 days, by which time skin degradation introduces yet another problem. The values of M/M_{true} quoted in Table 2 for allyl oestrenol were obtained from experiments run for about 15 days. They are therefore given without error estimates and can only be regarded as approximations.

It seemed also of interest to use this approach to determine the net barrier effect of the various layers which make up whole human skin. Table 3 gives data for whole skin, stratum corneum, epidermis and dermis, for nandrolone. From this data it is clear for example, that the dermis presents a net barrier less, but not greatly less, than the stratum corneum so far as the diffusing fraction is concerned. In the occluded situation, where the penetration fraction tends to a value of 1, and where drug penetration to the base of the dermis is of interest, the diffusion barrier presented by the dermis should not be neglected, at least for the case of nandrolone.

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Separation of the epidermal sheet by dispase

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SUMMARY

Dispase is a bacterial neutral protease which is obtained from the culture filtrate of *Bacillus polymyxa*. After 24-h treatment of the human skin with 500 and 1000 U/ml dispase, the epidermal sheet was easily peeled from the dermis, and its undersurface retained rete ridges. Electron microscopic observation showed that the basal surface was composed of cells with numerous slender villi and cytoplasmic projections. Although the intercellular spaces of the spinous as well as the basal layers were wide, all desmosomes were intact with their accompanying tonofilaments. An epidermal cell suspension was obtained by incubating the dispase-separated epidermal sheet in trypsin solution for 15 min and the viability of the cells was more than 95% as measured by the trypan blue dye exclusion test. A culture of keratinocytes was established by seeding the dissociated cells in the usual way.

For the cultivation of epidermal keratinocytes, it is desirable to obtain a suspension of viable cells which are not contaminated by dermal fibroblasts. Medawar (1941) separated a pure epidermal sheet by tryptic digestion of thin skin slices, and since then trypsin has been widely used for the separation of an epidermal sheet and its dissociation into a cell suspension (Wheeler, Camby & Cawley, 1957; Cruickshank, Cooper & Hooper, 1960; Briggaman *et al.*, 1967). However, trypsinization also causes loosening of the intercellular connection of the epidermal keratinocytes, and vigorous washing of the isolated epidermal sheet to remove the contaminating dermal fibroblasts also dissociates the keratinocytes. Slight modification of the conditions of trypsinization also causes separation at the suprabasal layer, leaving the proliferative population of keratinocytes on the dermis (Regnier, Delescluse & Prunieras, 1973). In electron micrographs, hemidesmosomes were shown to be more resistant than desmosomes to trypsin (Prunieras, 1979). These disadvantages led us to test other enzymes.

Dispase, a bacterial neutral protease produced by aerobically culturing a strain belonging to *Bacillus polymyxa*, was found to disperse mammalian tissue and cells (Matsumura *et al.*, 1975a; Irie, 1976). In contrast to trypsin, this enzyme is active in the presence of serum and Ca^{2+} and

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Mg^{2+} ions. Since dispase is very low in cytotoxicity, and stable during incubation for several days, L cells were viable and kept in monodisperse suspension in the presence of dispase (Matsumura *et al.*, 1975a). Matsumura *et al.* have tested the action of dispase on several strains of cells and differentiated them into three groups: (1) those detached from a glass surface and dissociated into single cells (L and CHO cells), (2) those detached but hardly dissociated (HeLa, JTC-16 and RTC-10 cells), and (3) those originally detached and dissociated without the aid of protease (FM3A). Those belonging to group 2 are cells of epithelial origin (Matsumura *et al.*, 1975b). Dispase has been successfully used to detach a sheet of cultured keratinocytes from the culture dish to obtain an epidermal sheet suitable for grafting (Green, Kehinde & Thomas, 1979).

By using dispase, we have successfully isolated the epidermal sheet of human skin from the dermis without dissociation of keratinocytes. A suspension of epidermal cells suitable for cultivation was then obtained by trypsinization of the isolated epidermal sheet.

MATERIALS AND METHODS

Chemicals

A crystalline preparation of dispase was purchased from Godo Shusei Co. (Matsudo, Chiba, Japan), and was dissolved in phosphate-buffered saline, Eagle's minimal essential medium (Eagle MEM) or Eagle MEM supplemented with 20% fetal bovine serum. Eagle MEM and 0·25% trypsin (Difco) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline were obtained from the Institute of Microbiology of Osaka University. Fetal bovine serum was purchased from Flow Laboratory.

Epidermal and dermal separation and dissociation of epidermal sheet

Human skin was obtained at plastic surgery, and cut into pieces measuring 3 × 5 mm. These were soaked in the solution of dispase at concentrations of 500 and 1000 U/ml. The dispase treatment was performed at 4°C, as in our usual method for the separation of the epidermal sheet by trypsin (Kitano & Okada, 1981). After the separation from the dermis, the epidermis was washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and incubated in a 0·25% trypsin solution for 15 min. An epidermal cell suspension was obtained by dissociating the epidermal sheet by shaking in the culture medium. The number of basal cells was determined by the criteria of Vaughan & Bernstein (1971). Viability of the cells was measured by the trypan blue dye exclusion test.

Culture of keratinocytes

The culture was initiated by seeding 8×10^5 basal cells suspended in 4 ml of culture medium into a 60 × 15 mm plastic tissue culture dish. This was incubated in an atmosphere of 95% air and 5% CO_2 at 37°C. The medium consisted of Eagle MEM supplemented with 20% fetal bovine serum (Kitano & Okada, 1981).

Electron microscopy

For electron microscopy the separated epidermal sheet was fixed in 2·6% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) for 30 min at 4°C. After washing in 0·1 M phosphate buffer, the sample was then postfixed in 1% osmium tetroxide in 0·1 M phosphate buffer (pH 7·4) for 1 h. The specimen was then dehydrated in an ethanol series, and embedded in Epon 812. Sections were cut with an LKB ultramicrotome, and stained with saturated uranyl acetate solution. The sections were observed with the Hitachi HU-12 electron microscope.

RESULTS

Separation of epidermis

After 24 h treatment with 500 and 1000 U/ml dispase in phosphate-buffered saline, Eagle MEM or Eagle MEM supplemented with 20% fetal bovine serum at 4°C, the epidermal sheet was easily peeled from the dermis by pulling with a fine forceps. These solvents did not influence the effectiveness of dispase, although the separated epidermal sheet was apt to be mucinous when phosphate-buffered saline was used. The thickness of the skin did not affect the result very much. When full-thickness skin with some subcutaneous adipose tissue was used, the epidermal sheet could be separated after somewhat prolonged treatment in dispase solution.

The undersurface of the separated epidermal sheet retained rete ridges (Fig. 1), and hair follicles with sebaceous glands and eccrine sweat glands were seen to be almost in their undistorted shapes. Separation of epidermis and dermis was observed at the basal zone in the routine histological section. Electron microscopic observation of the isolated epidermal sheet is shown in Fig. 2. The basal surface was composed of the cells with numerous slender villi and projections of the cytoplasm. Half desmosomes could not be identified. The intercellular spaces of the spinous as well as the basal layers were wide, but all desmosomes observed were intact with their accompanying tonofilaments (Fig. 2 inset), and the intercellular connection was not disrupted.

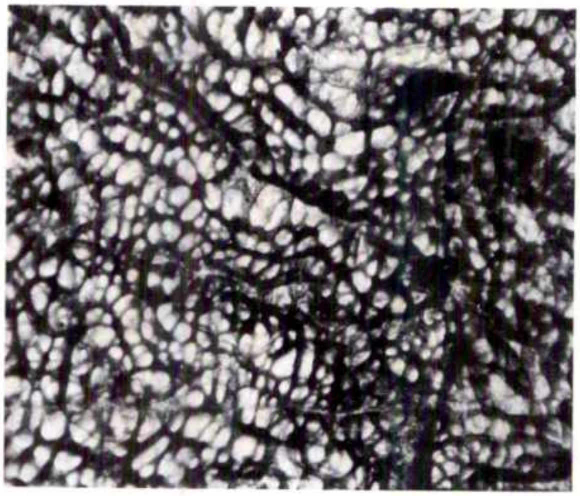

FIGURE 1. The undersurface of the epidermis split by digestion with dispase. The pattern of rete ridges was retained (× 20).

Dissociation and cultivation of keratinocytes

The dispase-separated epidermal sheets were incubated in 0·25% trypsin solution for 15 min. An epidermal cell suspension was obtained by dissociating the epidermal sheets by mechanical agitation. The viability of cells was more than 95%, measured by the trypan blue dye exclusion

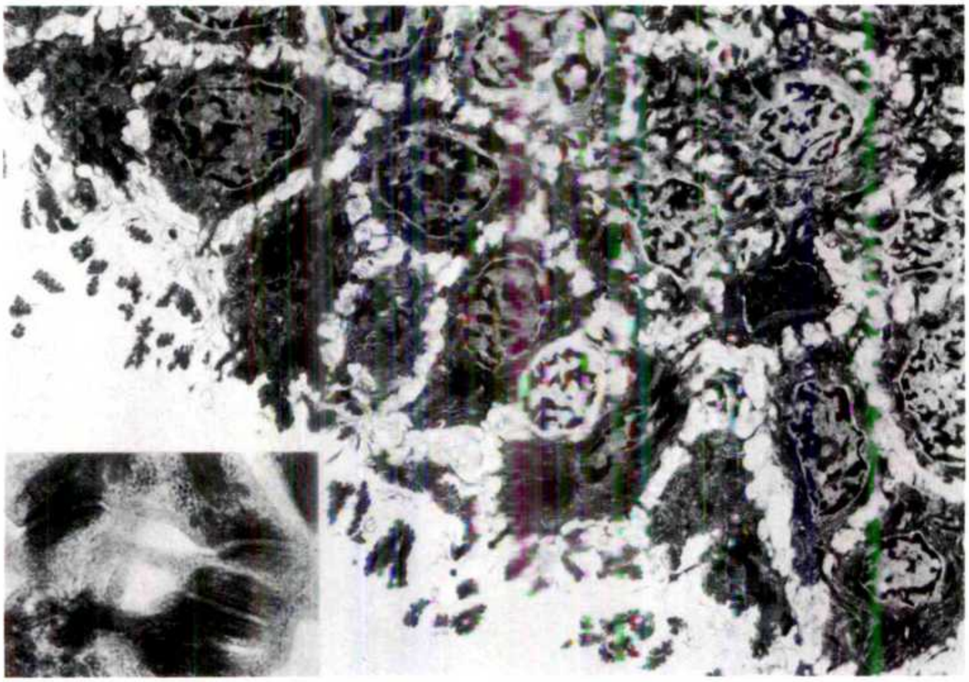

FIGURE 2. Electron micrograph of the isolated epidermal sheet after dispase digestion. The split occurred above the basement membrane. The intercellular spaces are wide, and numerous villi and projections of cytoplasm are observed (× 3096). Inset shows preservation of desmosomes without distortion (× 24,650).

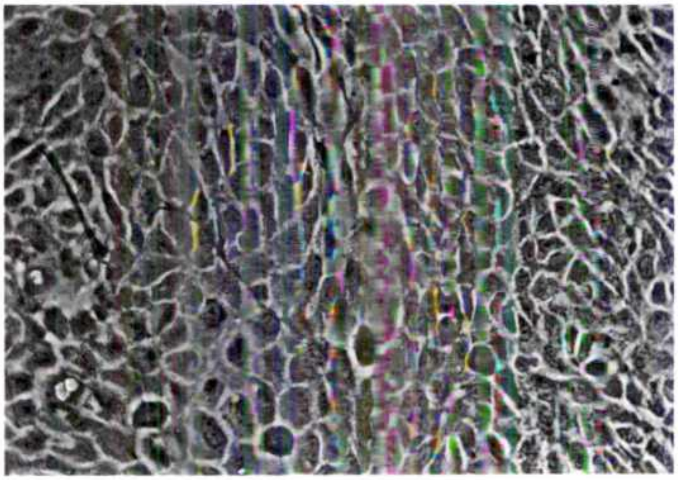

FIGURE 3. A culture of the keratinocytes. The cells proliferate to cover the culture surface almost completely. Mitotic figures are numerous. 13-day culture (× 150).

test. The culture was initiated by inoculation of 8×10^5 basal cells into a 60×15 mm dish, and followed the same course as the culture of keratinocytes obtained after trypsin treatment (Kitano & Okada, 1981). The keratinocytes attached to the bottom of a plastic culture dish singly or in small aggregates. Spindle or tripolar melanocytes were frequently observed. Within a few days the cells migrated around, and connected with each other to form a sheet composed of tightly packed polygonal cells. After 10 days of cultivation the proliferation of the cells became active, and the keratinocytes covered the culture surface completely (Fig. 3). Outgrowth of fibroblasts was never observed.

DISCUSSION

Previously trypsin has been almost the only enzyme used for isolating and dispersing the epidermis. Omar & Krebs (1975) made a comparative study on the effectiveness of pancreatic enzymes in epidermal separation, and found that crude lipase was more effective than crude trypsin (Difco), crude trypsin (Sigma) and pancreatin. A novel neutral protease, dispase, is produced by bacteria, and can be purified and crystallized. It is inhibited by neither serum nor Ca^{2+}, and its cytotoxicity is very low (Irie, 1976). Confluent cultures of keratinocytes have been treated with dispase, and the cultured epithelium has been detached from the Petri dish surface without dissociating the cells (Green *et al.*, 1979). We have now isolated the epidermal sheet *in vivo* from the dermis without dissociating the cells. The isolated epidermal sheet was then trypsinized to make a cell suspension suitable for cultivation.

A similar result can be obtained by the use of collagenase (Hentzer & Kobayashi, 1978). Split thickness human skin is incubated in 2 mg/ml clostridial collagenase for 3 h at 37°C, and epidermis is released from dermis as a single sheet. Collagenase does not separate the desmosomal connection between keratinocytes (Kobayashi, Hentzer & Asboe-Hansen, 1977). The epidermal sheet is then treated with trypsin-EDTA to be dispersed into cell suspension. When full thickness skin is used, it is necessary to incubate for 16 to 24 h in collagenase. No growth of epidermal cell is obtained from specimens incubated for 6 h or more. When we use 0·25% trypsin (Difco) solution, the epidermal sheet cannot be released from full thickness skin after 24 h treatment at 4°C (Kitano & Okada, 1981). The thickness of the skin does not seem to be important when dispase is used. After 24 h treatment with 500 U/ml dispase, the dermis was not fragmented and contamination of fibroblasts into the suspension of epidermal cells was minimal. The viability of the dissociated cells was more than 95%, and the culture followed the usual course of proliferation, stratification and keratinization.

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The importance of elastic fibres in skin lymphatics

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SUMMARY

Elastic fibres lie in intimate contact with dermal lymphatics. Demonstration of elastic by an orcein stain enables lymphatics to be identified and differentiated from blood capillaries. The finding of elastic suggests it plays an important role in the normal functioning of lymphatics.

Lymphatics transport protein and cells together with approximately 20% of tissue fluid from the tissues to draining lymph nodes; the fluid is no more than a solvent for this particulate matter and the main function of lymphatics, in partnership with macrophages, is protein removal. Antigen, together with macrophages and Langerhans cells, requires the lymphatic to convey it to the lymph node for lymphocyte recognition.

Lymphatics are potentially large structures because of their wide lumen, but they are not obvious in routine sections as they are invariably collapsed if they have been functioning effectively. Only when distended can they be seen readily, and even then differentiating them from blood vessels can be difficult. A distended lymphatic (Fig. 1) shows the characteristically thin walls when compared to the thicker wall of a nearby blood vessel. Ultrastructurally the wall of a lymphatic is attenuated with open junctions between the overlapping endothelial cells (Fig. 2); collagen fibrils and elastic, with its dark staining and amorphous consistency, are seen adjacent to the endothelial cells (Braverman & Yen, 1974).

The function of the lymphatic and the means by which large protein molecules enter the vessel depend upon the compliance of the surrounding tissues. Movement of these tissues by compression and stretch brought about by such physical forces as massage, muscle pump and vibration, manipulates the lymphatic in such a way as to open junctions. It has been assumed that it is collagen which provides the framework on which the lymphatic functions (Leak & Burke, 1968), but it may be that elastic is equally important, particularly with regard to stretch and recoil of the vessel.

We therefore undertook to study elastic and its relationship to the lymphatic in (1)

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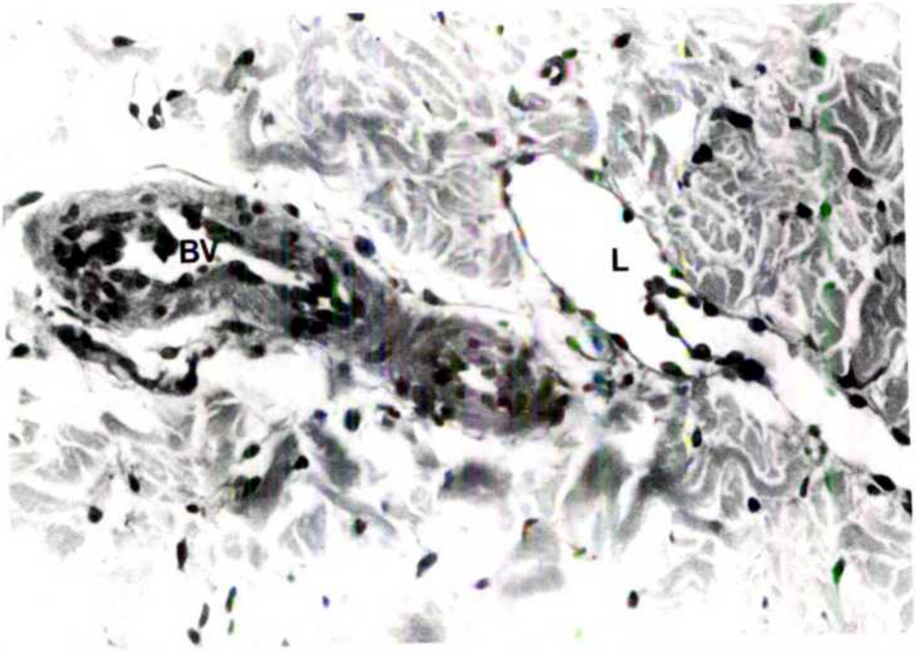

FIGURE 1. Pig skin. A distended lymphatic (L) shows characteristically thin walls compared with the thicker wall of a nearby blood vessel (BV) (× 420).

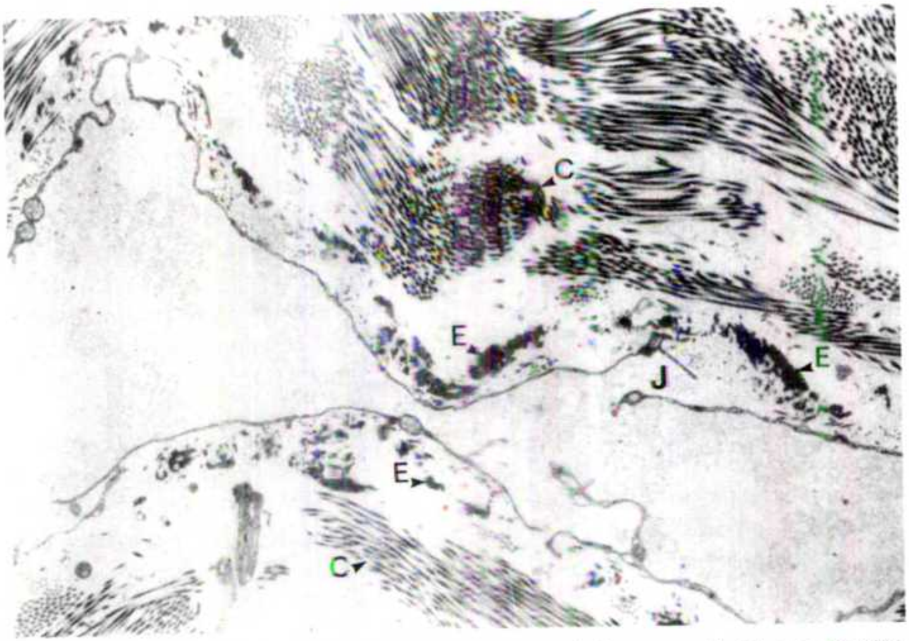

FIGURE 2. Human skin. Ultrastructurally the wall of a lymphatic is attenuated with open junctions (J) between the overlapping endothelial cells. Elastic (E) lies in close proximity to the endothelium with collagen fibrils (C) beyond (× 7161) (Courtesy of I.M.Braverman & A.Yen).

lymphoedematous pig skin, where distended lymphatics were readily identified, and (2) normal human skin.

MATERIALS AND METHODS

Pig skin

The lymphatics of an area of pigskin flank were severed while maintaining the blood supply via a vascular pedicle. Disulphine blue (I.C.I) was injected intravenously. The dye remained in the tissues of the flap, indicating failure of lymphatic drainage. Biopsies were taken from both the lymphoedematous flap and normal skin. Paraffin wax sections were stained with haematoxylin and eosin (H & E), and orcein.

Human skin

Workers have expressed the belief that blood vessel endothelial cells show positive staining for Factor VIII-related antigen (FVIII-RAG), while lymphatics show no such staining (Mukai, Rosai & Burgdork, 1980). In the hope that this may also prove to be a useful way of

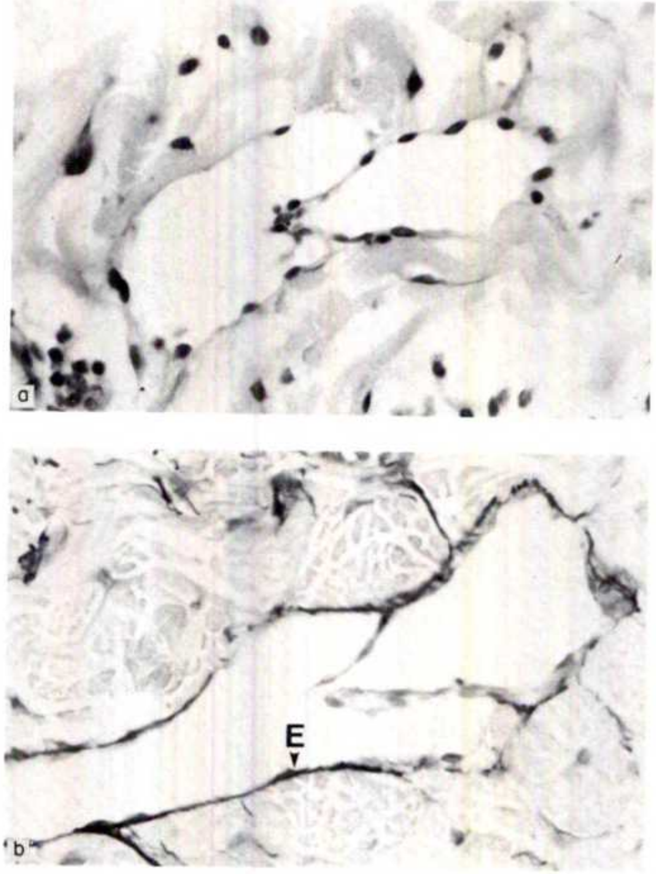

FIGURE 3. (a) Pig skin (H & E). Dilated lymphatic within lymphoedematous flap (× 700). (b) Pig skin (orcein). Elastic (E) is seen outlining the same lymphatic (× 560).

differentiating between blood vessels and lymphatics, biopsies were examined using a peroxidase/anti-peroxidase (PAP) technique (Burns, 1975). Biopsies were taken from normal human skin and embedded in paraffin wax. Consecutive sections were stained with H & E, orcein, and the PAP method for FVIII-RAG.

RESULTS

Pig skin

In lymphoedematous pig skin dilated lymphatics could be readily seen in H & E sections (Fig. 3a). Orcein-stained sections showed elastic outlining these lymphatics to an extent that actually highlighted them (Fig. 3b). Even collapsed lymphatics from normal pig skin could be visualized with elastic fibres extending several millimetres through the mid-dermis (Fig. 4). Only lymphatics would extend to these lengths; blood capillaries and venules twist and turn through the skin so that much shorter lengths are observed in a 6 μ section. With such attenuated endothelium and obliterated lumen this lymphatic would not be identified in H & E sections. Small blood vessels did not possess any elastic unless they were large enough to have acquired an internal elastic lamina.

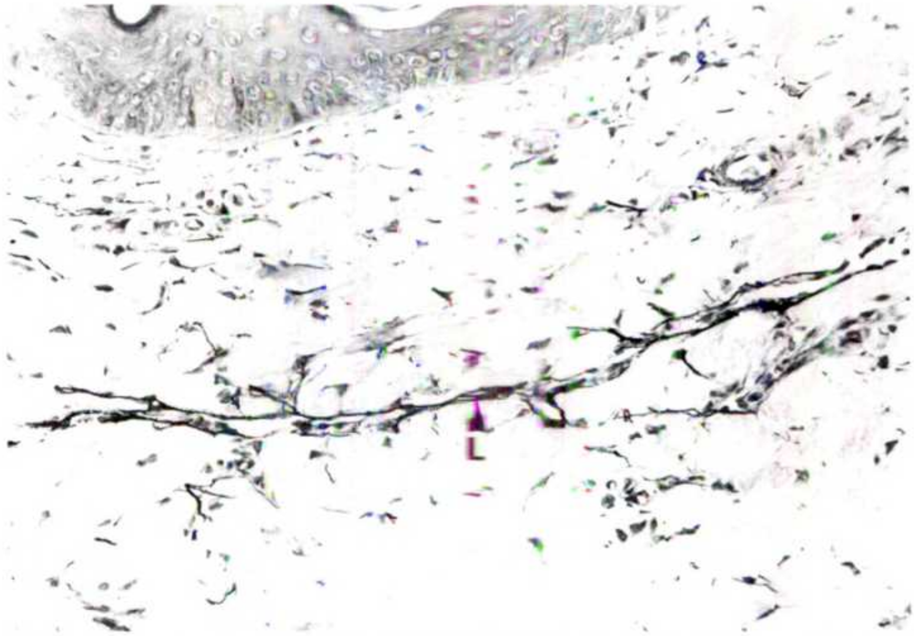

FIGURE 4. Pig skin, normal (orcein). A collapsed lymphatic (L) surrounded by elastic and extending several millimetres through the mid-dermis (× 210).

Human skin

Biopsies of normal human skin showed identical findings, with elastic outlining lymphatics and thereby differentiating them from blood capillaries. Many blood vessel endothelial cells showed positive staining for FVIII-RAG, but lymphatics were consistently negative (Fig. 5).

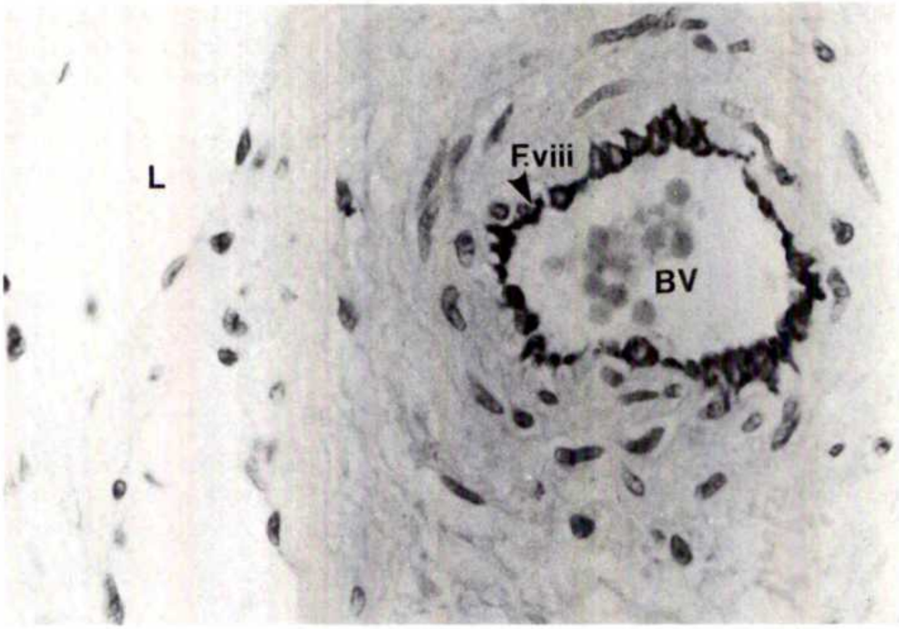

FIGURE 5. Human skin. Blood vessel endothelium shows positive staining for Factor VIII-related antigen (Fviii) but lymphatic endothelium shows negative staining. Blood vessel = BV, lymphatic = L (× 1400).

DISCUSSION

In normal pig and human skin an orcein stain demonstrated elastic surrounding skin lymphatics to an extent that actually highlighted the vessels, and helped to differentiate them from blood vessels.

Until now, except for a brief mention in a review of lymphatics of the skin (Ryan, 1978), there has been no comment on the relationship between elastic fibres and lymphatics. Orcein stains are used routinely at light microscopy level to show up blood vessels, but careful observation reveals that dermal blood vessels do not possess elastic unless they are large enough to have acquired an internal elastic lamina. Casley-Smith & Florey (1961) described bundles of collagen fibres investing the lymphatic capillaries. Leak & Burke (1968) observed elastic fibres in close proximity to the lymphatic capillary wall but made no further comment. They described anchoring filaments in association with lymphatic endothelial cells in guinea-pig skin and suggested that during the inflammatory state the walls of the lymphatic capillary are pulled upon by the anchoring filaments and collagen bundles, thereby causing widening of the lumen and allowing entry of material into the lymphatic. Beyond these observations, no relationship between the elastic fibres and the lymphatic capillary has been promoted. The finding of elastic to the extent we have demonstrated suggests that it is fundamentally important for the normal function of the lymphatic. If this is so, to what extent does abnormal or destroyed elastic contribute to disease morphology by way of lymphatic malfunction? In rosacea, for instance, Marks & Harcourt-Webster (1969) stressed that the only consistent histological feature was solar elastosis. Sections of rosacea stained with orcein reveal no elastic surrounding dermal lymphatics within the area of elastosis. Does this loss of elastic explain the lymphoedema in this condition?

In leprosy, elastic is destroyed in the granuloma. Lymphatics are the exit channel for the macrophage and it is possible that the loss of elastic in the early lesion of the neurovascular bundle impairs lymphatic function, thereby preventing clearance of macrophage and processing of antigen.

In mycosis fungoides, elastic is destroyed very early in the disease. Since lymphatics are necessary for transporting antigen to the lymph node for lymphocyte recognition, is their malfunction important in the impaired processing of immunological information?

While FVIII-RAG was an inconsistent marker of blood vessel endothelium, it was consistently negative in lymphatics. We did not demonstrate the 90–95% positive staining of blood vessel endothelium described by other workers. Nevertheless, we did find it a useful adjunct in helping to distinguish between lymphatic and blood vessels.

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A new method for the determination of steroid sulphatase activity in leukocytes in X-linked recessive ichthyosis

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SUMMARY

The diagnosis of X-linked ichthyosis can now be reliably established by using a non-radioactive method to detect steroid sulphatase deficiency in leukocytes. This new method yields the same results with leukocytes as with cultured fibroblasts. The second type of microsomal arylsulphatase previously described in cultured fibroblasts is also present in leukocytes.

X-linked recessive ichthyosis (XRI) is one of the manifestations of the X-linked steroid sulphatase (SS) deficiency syndrome (Jöbsis *et al.*, 1976; Koppe *et al.*, 1978; Shapiro *et al.*, 1978a; De Groot *et al.*, 1980). SS is one of the microsomal arylsulphatases (MAS), a group of enzymes also known as arylsulphatase C (Van der Loos *et al.*, 1979, 1981). The mechanism by which the deficiency produces ichthyosis has not yet been elucidated. The inability to hydrolyze cholesterol sulphate is probably an important factor (Epstein, Krauss & Shackleton 1981). In the other types of ichthyosis, SS activity is normal (Kubilus, Tarascio & Baden, 1979; Meyer *et al.*, 1979; Shapiro *et al.*, 1978b).

MAS activity can be demonstrated histochemically in the granular layer of the normal epidermis but it is not present in the epidermis of XRI patients (Jöbsis *et al.*, 1976; Koppe *et al.*, 1978; De Groot *et al.*, 1980; Meyer *et al.*, 1982). In autosomal dominant ichthyosis (ADI) Meyer *et al.* (1982) found that this activity is usually present but De Groot *et al.* (1980) had more variable results in ADI, finding activity in only some of their cases. Furthermore Meyer *et al.* (1982) confirmed our observations with regard to the great variation in histochemical staining results found with skin from different body areas. These divergent results render this

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histochemical method on cryostat sections unsuitable for diagnostic purposes. Neither can this differentiation between XRI and ADI be made by determining the dehydroepiandrosterone sulphate (DHEAS) level in serum (Ruokonen *et al.*, 1980).

SS activity in cultured skin fibroblasts can be measured using a radioactive method. In XRI the activity is virtually absent whereas in ADI the activity is as high as in healthy controls (Shapiro *et al.*, 1978b; Kubilus *et al.*, 1979; Meyer *et al.*, 1979). The diagnostic method is reliable but laborious and can only be performed in specialized laboratories. In order to avoid these disadvantages we have investigated SS activities using a non-radioactive method in extracts from leukocytes which are easily obtained.

METHODS

Patients and controls

XRI (seven cases), ADI (five cases) or autosomal recessive ichthyosis (ARI) (one case) was diagnosed by one of us (De Groot) by clinical appearance, microscopy and family history. In some cases ophthalmological examination was also performed. The age of the subjects ranged from 11 to 82 years. The controls (CO) consisted of ten healthy males and ten healthy females (age range 11–45 years).

Materials

The following chemicals and reagents were used: Miranol H2M from Miranol Chemical Company (Irvington, U.S.A.); the chemicals used in polyacrylamide gel electrophoresis (PAGE) from Bio-Rad (Richmond U.S.A.); potassium 6-bromo-2-naphthyl sulphate (BNS), sodium dehydroepiandrosterone sulphate (DHEAS), Fast Blue B and DL-dithiothreitol (DTT) from Sigma (St Louis, U.S.A.); 7-[H^3](N)-DHEAS (ammonium salt) 24 Ci/mmol from NEN (Boston, U.S.A.). Ham's F10 and fetal calf serum were obtained from Flow Laboratories (Irvine, Scotland). All chemicals used were of analytical grade.

Leukocytes were isolated (Wyss, Koster & Hülsmann, 1971) from 10 ml heparinized blood within 3 h of venepuncture. Erythrocytes were lysed in a medium consisting of 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0·1 mM EDTA. The leukocytes were suspended in 1·1 ml Tris-HCl buffer (pH 7·2, 100 mM). The protein content was determined according to Lowry *et al.* (1951). The leukocyte suspension was then diluted to a protein content of 2·5 mg/ml. Then 200 μl Miranol H2M 10% (v/v) was added to 1 ml diluted leukocyte suspension and incubated at 37°C for 30 min. This extract was dialysed (4°C) overnight against electrode buffer containing 10^{-4} M DTT and 0·5% Miranol H2M. Cell debris was removed by centrifugation at 20,000 ***g*** (4°C). In order to determine the activity of solubilized SS, PAGE was applied. Two 500 μl samples (1·25 mg protein) from the supernatant were applied to 7·5% polyacrylamide gels, pH 7·5 (Van der Loos *et al.*, 1981). After PAGE, MAS activity was demonstrated in one gel using a procedure based on a histochemical method (Van der Loos *et al.*, 1981). During this histochemical reaction, the substrate BNS is hydrolyzed by MAS; an insoluble purple dye is formed by an azo-coupling reaction of the desulphatized 6-bromo-2-naphthol with Fast Blue B. In order to check the result obtained with this method in a gel, SS activity was assayed in the second gel using [^{3}H]DHEAS as substrate (Van der Loos *et al.*, 1981). After PAGE, this gel was cut into thirty 2-mm slices, each slice being put into a small tube. To each tube 150 μl Tris-HCl buffer (pH 7·2, 100 mM) was added. The tubes were shaken overnight at 4°C and SS activity was measured in the buffer. In order to measure SS activity separately in lymphocytes and granulocytes, these cells were isolated from 30 ml heparinized venous blood (Bøyum, 1964) and counted in a Bürker chamber. SS activity was determined in 2 × 10^6 cells and expressed in pmol DHEA per hour per cell.

Skin fibroblasts were cultured from one XRI patient and two ADI patients and one healthy control in Ham's F10 medium including 20% fetal calf serum.

RESULTS

PAGE of the leukocyte extracts from all controls, ADI patients and the ARI patients, followed by histochemical demonstration of MAS activity, resulted in two purple bands with R_F values of 0·49 ± 0·02 and 0·58 ± 0·02 (Fig. 2). The same procedure performed on leukocyte extracts from XRI patients yielded only the band at R_F 0·58. Identical results were obtained when the

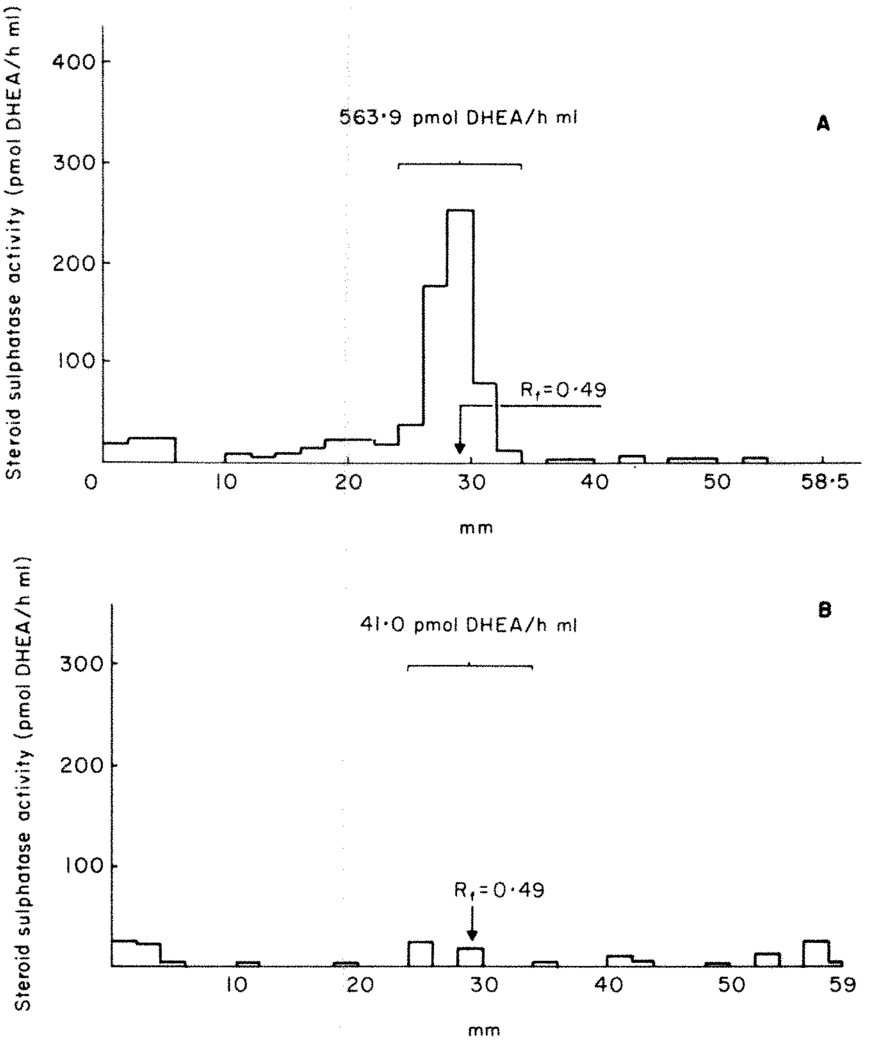

FIGURE 1. Graphs showing the SS activity in 2 mm gel slices after PAGE of leukocyte extract samples. A = female control; B = XRI patient.

determination was carried out on extracts from fibroblasts: two bands in the control patients and ADI patients and only the 0·58 band in the XRI patients.

SS activity in the gel was found in only five of the slices, at $R_F = 0{\cdot}49$. The total activity was calculated by adding the values obtained from five slices (Fig. 1). The SS activity of leukocytes from both patients and controls is shown in Fig. 3. The mean SS activity and standard deviation in the leukocytes of the female and male controls was 511·4 ± 63·5 and 342·7 ± 70·6 pmol

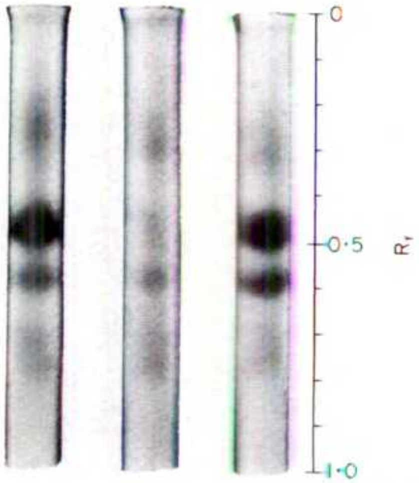

FIGURE 2. PAGE of leukocyte extract samples followed by the histochemically based demonstration of MAS activity; male control (left), XRI patient (middle), male ADI patient (right).

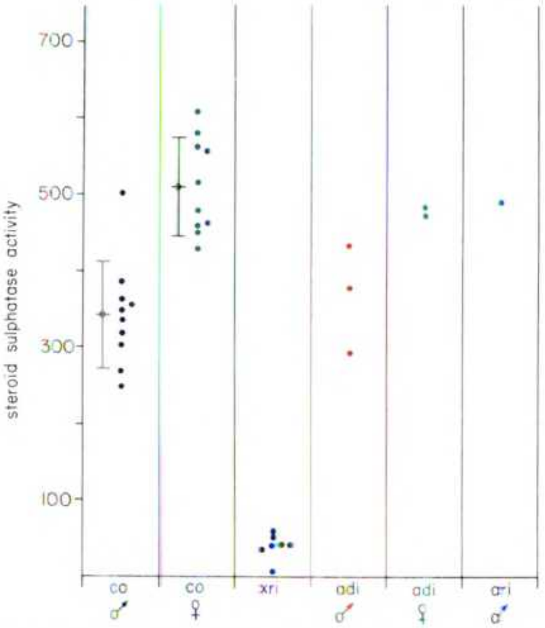

FIGURE 3. SS activity in leukocyte extracts. co = control, xri = X-linked recessive ichthyosis, adi = autosomal dominant ichthyosis, ari = autosomal recessive ichthyosis.

DHEA/h/ml respectively. The leukocytes of patients with XRI showed an SS activity lower than 58·7, but female and male patients with ADI showed enzyme activities within normal limits (Fig. 3). The activity was independent of the age of the subject. SS activity of the leukocytes was not influenced within 6 weeks when stored at −20°C; suspensions were not investigated after this period. SS activity expressed in pmol DHEA/h/cell was almost twice as high in lymphocytes as in granulocytes measured in four controls.

DISCUSSION

From these results we conclude that SS deficiency can be diagnosed in leukocytes. Ruokonen & Oikarinen (1981) have already demonstrated that the need to culture fibroblasts can be avoided by measuring SS activity directly in skin biopsies. The use of leukocytes, however, has obvious advantages. Epstein & Leventhal (1981) have also investigated SS activity in leukocytes. Although our method differs, our results are in accordance with those of Epstein (De Groot, Jöbsis & Van der Loos, 1982). With both methods, higher SS activity values were found in females than in males and SS activity was virtually absent in XRI patients and normal in ADI and ARI patients. Though the SS activity in lymphocytes is twice that in granulocytes, the usual variation in the composition of peripheral leukocytes will not detract from the diagnostic significance of this method with regard to the types of ichthyosis investigated. Furthermore, the PAGE procedure followed by the histochemically based demonstration of MAS activity provides a non-radioactive method of distinguishing between XRI and other types of ichthyosis. While this work was in progress, Epstein *et al.* (1981) demonstrated that serum components have different properties in XRI and in normal males but the differential diagnostic value of this interesting observation, also based on a non-radioactive method, was not indicated. Recently Meyer *et al.* (1982) described a diagnostic non-radioactive method based on the capacity of leukocytes to desulphatize the artificial substrate 4-methylumbelliferylsulphate. Since this method does not involve electrophoretic procedures, it is not clear whether it exclusively registers the activity of SS or whether other sulphatases influence these findings.

In the histochemically stained gels two purple MAS activity bands were demonstrated. The one at R_F 0·49 represents SS since the activity peak of this enzyme in the [^{3}H]DHEAS assay is also found at R_F 0·49, whilst at R_F 0·58 SS activity is not detectable (Fig. 1a). The metabolic function of the 0·58 MAS enzyme, already discovered in cultured fibroblasts, is not known (Van der Loos *et al.*, 1981). We do not even know which natural substrate(s) it can hydrolyse *in vitro*. At present we are investigating the variation in staining intensity of the two bands in extracts from various organ types, including skin.

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The correlation between surface microscopy and dermal histology in tetradecane-induced comedones in rabbit ear skin

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SUMMARY

Three concentrations of tetradecane and liquid paraffin were applied daily for 4 weeks to the external ear skin of rabbits, and the average diameter of the follicular lesions was measured under the Leitz MZ surface microscope. These observations showed that the increase in the size of follicular lesions with tetradecane was dose-dependent and the maximum diameter was reached on the 14th day. Serial biopsies were also taken for routine histology, and undiluted tetradecane was shown to produce follicular hyperkeratosis, microcomedones, closed comedones and open comedones. A good correlation was obtained between the surface observations and the histological findings. The surface microscope provides a simple and convenient *in vivo* method for evaluating comedo formation.

The rabbit ear model (Kligman & Katz, 1968; Fulton *et al.*, 1976; Woo-Sam, 1978) has been widely used for histological studies of comedogenesis. A new technique is described which gives good visualization of rabbit ear comedones at a high magnification without the need for a biopsy. In this study, the Leitz MZ surface microscope (Cunliffe, Forster & Williams, 1974) was used to clarify the correlation between the surface microscopical observations and the histological findings in tetradecane-induced comedones of the rabbit ear.

METHODS

The test chemicals were n-tetradecane (Kanto Chemicals, purity = 99.5%) and liquid paraffin (Nakarai Chemicals, $n_D^{20} = 1{\cdot}460–1{\cdot}470$).

Sixteen young male Japanese white rabbits were used. Undiluted tetradecane, 50% and 10% tetradecane in liquid paraffin, or liquid paraffin were applied to the ventral surface of the rabbit

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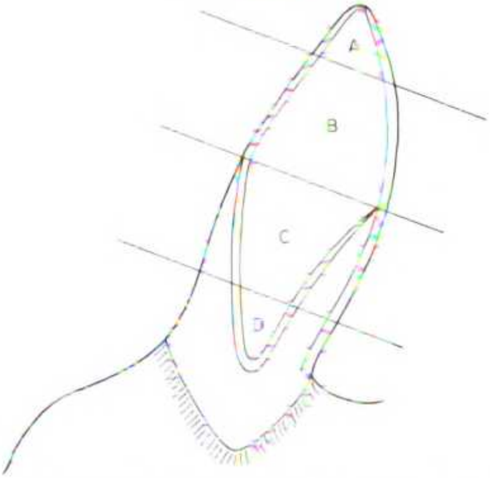

FIGURE 1. Application site (D) on the ventral surface of the rabbit ear, near the auditory canal.

ear, near the auditory canal (D area), using the tip of a glass rod (Fig. 1). The other ear was left untreated as a control.

The chemical was applied daily for 4 weeks on the ears of four rabbits and the diameters of ten follicular eruptions per ear were measured daily under the Leitz MZ microscope. Biopsies were also taken from each group on the 1st, 3rd, 7th, 10th, 14th, 21st and 28th days. These specimens were processed for light microscopy.

RESULTS

The daily application of tetradecane for 14 days to the external ear skin of rabbits induced good comedo formation. Under the Leitz MZ microscope, many erythematous indurated lesions with crusts in the centre were seen (Fig. 2).

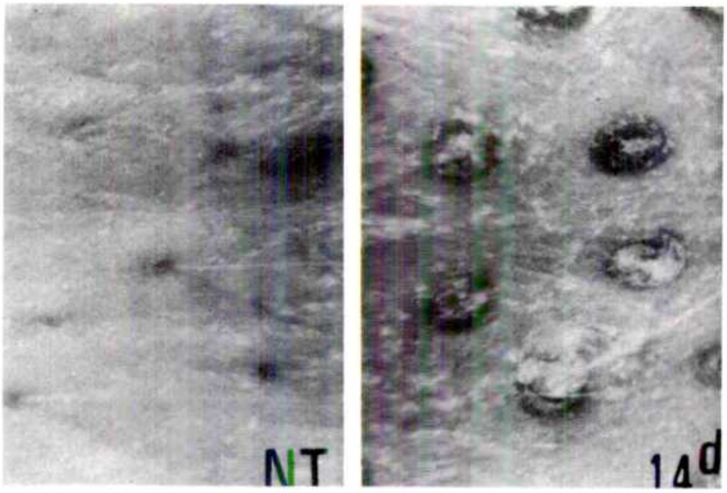

FIGURE 2. Photographs by the Leitz MZ surface microscope and Nikon camera. Left (NT): non-treated skin. Right (14^d): tetradecane-treated skin on 14th day.

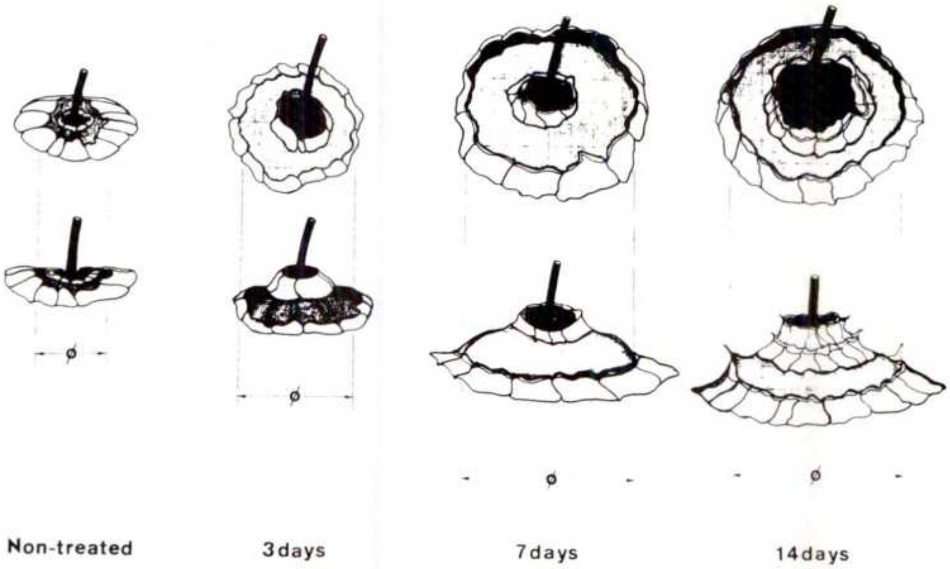

FIGURE 3. Serial changes of the average diameter of skin eruptions produced with tetradecane under the Leitz MZ surface microscope.

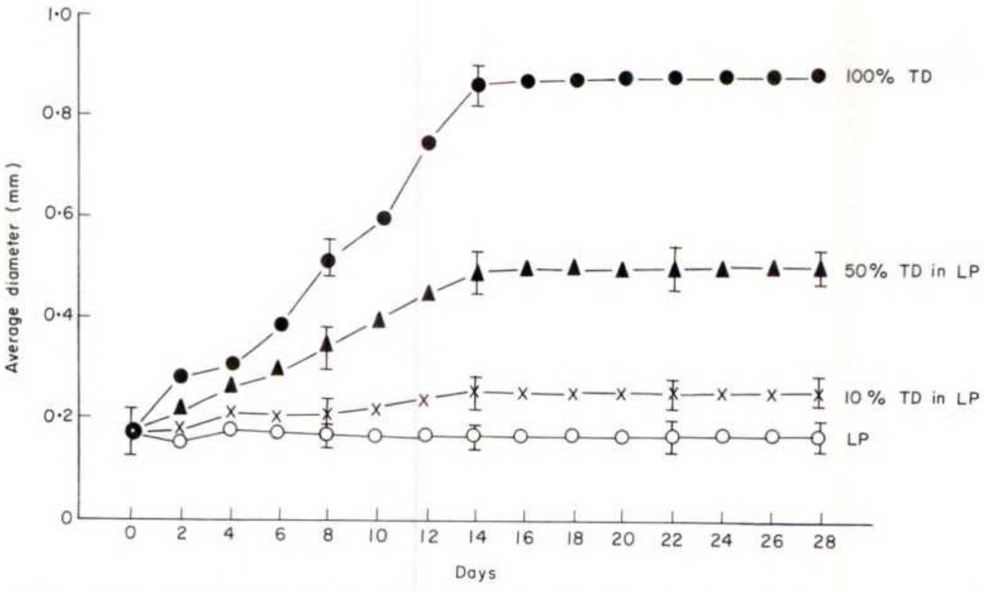

FIGURE 4. Comedogenicity of various concentrations of tetradecane. TD = tetradecane. LP = liquid paraffin.

Although it was necessary to determine the degree of follicular extension, it was not possible to measure the diameters of the pilosebaceous duct orifices of the lesions because of the presence of crusts. Therefore, the average diameter of the erythematous, indurated areas, which should be proportional in size to the follicular hyperkeratosis or comedones in the dermis, was employed (Fig. 3).

This observation showed that the increase in average diameter of follicular lesions induced with tetradecane was dose-dependent and the maximum diameter was reached on the 14th day. Liquid paraffin did not produce a follicular eruption (Fig. 4).

Histological serial changes of the pilosebaceous units of the rabbit ear treated with tetradecane are summarized in Fig. 5. Follicular hyperkeratosis and microcomedones were seen from the 1st to 3rd day, closed comedones from the 7th to 10th day and open comedones from the 14th to 28th day.

Marked hyperplasia and hyperkeratosis of follicular epithelium and a diffuse infiltrate of

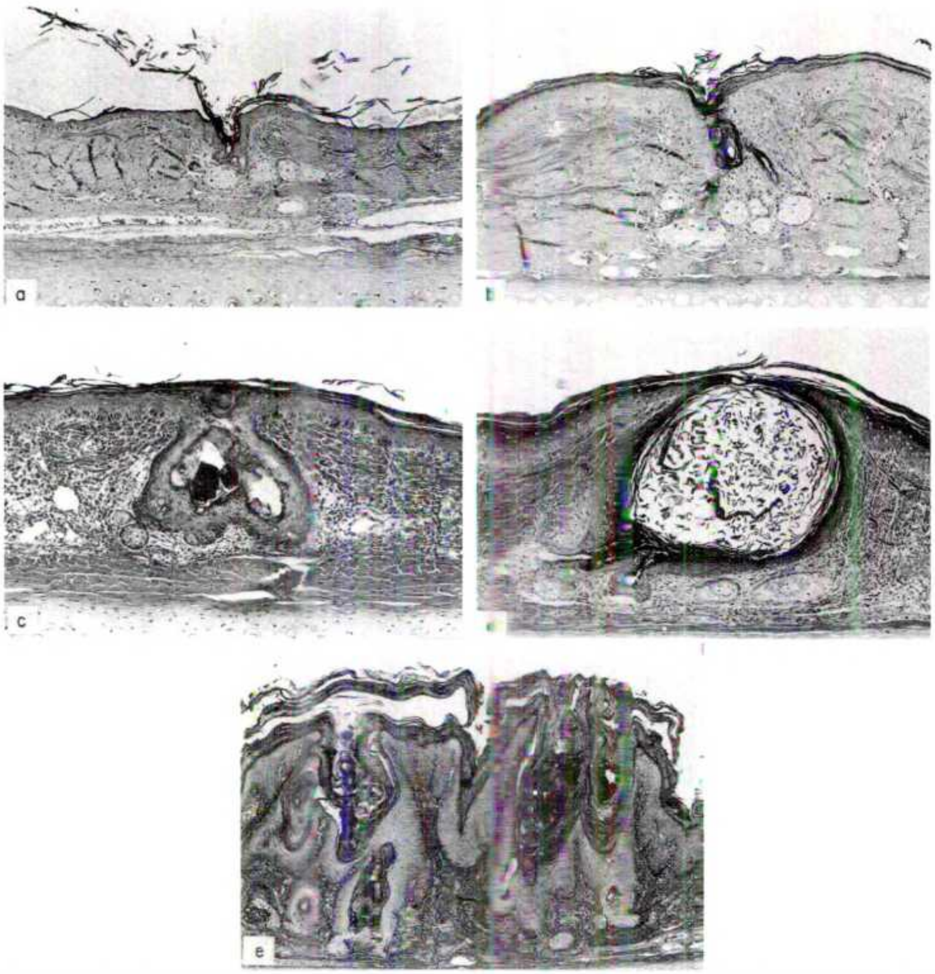

FIGURE 5. Histological serial changes in a pilosebaceous unit of the rabbit ear treated with tetradecane: (a) non-treated, (b) follicular hyperkeratosis on the 3rd day, (c) microcomedone on the 7th day, (d) closed comedone on the 10th day, (e) open comedone on the 14th day.

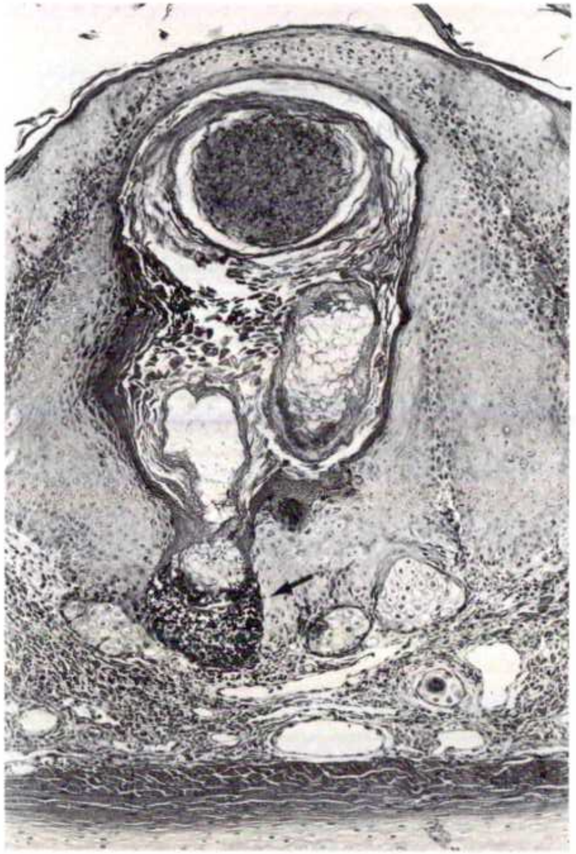

FIGURE 6. A considerable number of leucocytes (arrows) invaded the sebaceous glands treated with tetradecane on the 7th day.

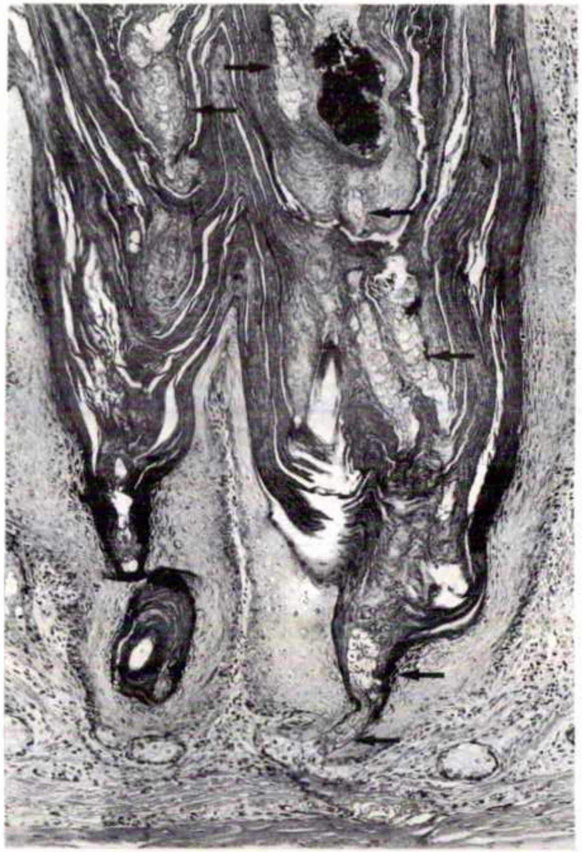

FIGURE 7. Eosinophilic sebaceous cells (arrows) undergoing holocrine secretion were increased in number and appeared to be pushed upward into the matured comedone on tetradecane treated skin at the 14th day.

mononuclear and polymorphonuclear cells around the hair follicles developed within 3 days. A few polymorphonuclear leucocytes invaded the thickened follicular epithelium, and a considerable number of leucocytes invaded the sebaceous ducts and also the sebaceous glands (Fig. 6).

In addition, marked proliferation of sebaceous cells was seen and eosinophilic cells undergoing holocrine secretion seemed to increase in number. Some of these cells appeared to be pushed upward into the mature comedone with an accumulation of infiltrated leucocytes (Fig. 7).

In this study, the average diameter of the erythematous, indurated lesions was used to evaluate comedo formation. It was necessary, therefore, to determine whether this diameter was really proportional to the size of the comedones in the dermis. Hematoxylin and eosin-stained sections were studied and the size of ten comedones was measured at each stage with the aid of a Nikon objective micrometer.

As shown in Fig. 8, a good correlation was obtained between the surface diameters of the lesions and the histological average size of the dermal follicular hyperkeratosis or comedones.

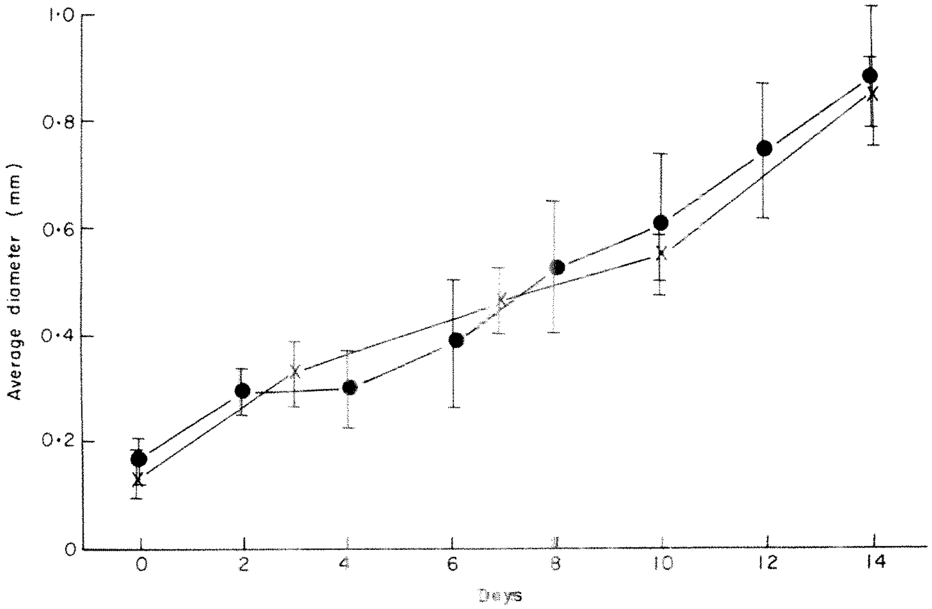

FIGURE 8. Relation between the surface microscopic average diameters of eruptions and the histological average sizes of follicular hyperkeratosis of comedones on tetradecane-treated skin. (●——●): Leitz MZ surface microscopic measurements. (×——×): Nikon objective micrometer measurements.

DISCUSSION

Recently, Kligman & Kwong (1979) proposed an improved rabbit ear model, in which excised tissue was immersed in water at 60°C for 2 min, yielding a sheet of epidermis with microcomedones attached, and the magnitude of the follicular hyperkeratosis was evaluated with a stereomicroscope. Although their whole mount technique is quick and convenient, it is noted that it does not avoid a biopsy and it is not quantitative.

We have now found a good correlation between the surface microscopic observations and the histological findings in tetradecane-induced comedones. This *in vivo* method, using the surface microscope, is a very simple and convenient method for evaluating comedo formation.

The histological changes we observed were in agreement with those reported by Kligman & Katz (1968), apart from the following observation. It was noteworthy that marked proliferation of sebaceous cells was seen and that eosinophilic cells undergoing holocrine secretion appeared to increase in number. Such marked proliferation of sebaceous cells might be an important factor in comedogenesis as well as the hyperkeratosis of follicular epithelium.

The pilosebaceous unit of rabbit-ear skin differs anatomically from that of human skin (Kligman & Katz, 1968). The sebaceous glands of the rabbit ear (Fig. 5a,b) had many small acini, each of which had an individual duct. The individual sebaceous duct fused with adjacent ducts and formed a common sebaceous duct, which entered the follicular infundibulum. These variations might be expected to produce reactions that differed from the reaction seen in human acne vulgaris, but the results obtained showed that tetradecane-induced rabbit ear comedogenesis was consistently associated with the characteristics of the three types of comedones (micro, closed and open) seen in human acne vulgaris. We therefore feel that this is an interesting model for studying morphologic changes in pilosebaceous units.

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Immune responses of patients with tinea imbricata

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SUMMARY

Tinea imbricata is a chronic dermatophyte infection caused by *Trichophyton concentricum* affecting large areas of the skin surface. Spontaneous improvement is unusual and relapse after apparently successful treatment is common. In this study in Papua New Guinea it was found that a high proportion of infected patients had immediate-type hypersensitivity (52%) or negative responses (46%) to intradermal trichophytin. The majority of patients failed to develop delayed-type hypersensitivity on skin testing or as assessed *in vitro* by leucocyte migration inhibition. However, 78% of patients investigated had antibody to *T. concentricum*. The relevance of T-lymphocyte hyporeactivity to persistence of the infection is discussed.

Tinea imbricata is the infection caused by the dermatophyte *Trichophyton concentricum*. It is confined to endemic areas in the Far East, the West Pacific and South America, although there are reports of scattered cases occurring elsewhere. Within these zones the disease may affect a large proportion of the population and it covers extensive areas of skin with concentric and lamellar scaling. The disease may be a great social burden to affected patients, who are less likely to marry, and it also causes intermittent severe irritation. In Papua New Guinea tinea imbricata is well recognized in lowland or coastal areas, (Schofield, Parkinson & Jeffrey, 1963) particularly in the East and West Sepik and the islands of Milne Bay Province where it is called grille or sipoma. It is also seen elsewhere, although less frequently.

Patients with tinea imbricata are often chronically infected from early childhood and the infection usually relapses after an initial response to local or systemic antifungal therapy. Spontaneous remissions are rare. These observations suggest that there is widespread susceptibility in the population and that effective immunity does not develop or is suppressed in the course of infection. This is in striking contrast to certain other dermatophyte infections in man such as cattle ringworm *T. verrucosum* where lifelong immunity appears to follow infection

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(Hall, 1966). In order to examine the immunological responses to *T. concentricum* infection we have studied a heavily infected population on Goodenough Island in Milne Bay Province, Papua New Guinea.

METHODS

Patients

One hundred and two patients were interviewed and examined at Bolubolu medical aid post on Goodenough Island. Scrapings were taken from each for confirmatory cultures and an assessment of the duration of disease and the extent of skin involvement was made. Blood samples were taken for leucocyte studies and antibody determinations from selected volunteers. Skin tests were also evaluated in infected individuals.

Antigens

Cytoplasmic antigens were prepared from *T. concentricum* NCPF 567 by a method described previously (Mackenzie, Proctor & Philpot, 1980). Seven-day-old mycelium grown in Sabouraud's broth was disrupted in a Dynomill (Basel) and the lyophilized supernatant reserved as antigen. This antigen was used in leucocyte migration studies and skin tests.

Intradermal tests

Lyophilized antigen was dissolved in 1 ml distilled water to give a concentration of 70 μg protein/ml as estimated by a protein binding method (Bradford, 1976). It was dialysed overnight against sterile distilled water and diluted 1:20 in sterile N-saline. Phenol (0·05%) was added. Positive skin test reactions could not be elicited in four non-sensitized guinea-pigs and in ten uninfected human subjects. However, delayed-type skin tests were elicited in four guinea-pigs sensitized 3 weeks previously with *T. concentricum* skin test antigen containing 70 μg protein in 1 ml Freund's complete adjuvant. Test sites showed a mean area of swelling of 36 mm^2 48 h after intradermal injection of 0·02 ml aliquots of antigen. Sensitized guinea-pigs also showed weak delayed-type skin reactions to commercial trichophytins (Bencard Ltd., U.K.) prepared from *T. rubrum* and *T. mentagrophytes* of 12·6 mm^2 and 16·9 mm^2 respectively.

Seventy infected individuals were injected with 0·02 ml of the intradermal antigen in the volar surface of the forearm or an adjacent site if the former was affected by the disease. Skin tests were examined at 20 min and 48 h. Immediate-type hypersensitivity was recorded in individuals showing weals with an accompanying flare over 8 mm in diameter. Delayed-type responses of 3–5 mm and over 5 mm in diameter were recorded. Ten non-infected control subjects on Goodenough Island and ten patients with a *T. rubrum* infection in the United Kingdom were also tested.

Leucocyte migration inhibition

The technique used has been described elsewhere (Hay & Brostoff, 1977). The following modifications were carried out. In each test three replicates were used. Plates were incubated overnight (24 h) in Eagle's minimum essential medium (Gibco, U.K.) containing 15% fetal calf serum, both in the presence and absence of antigen of *T. concentricum* at 35°C. Prior to carrying out the study the antigen was assessed for toxicity using peripheral blood leucocytes from eight non-infected human controls and for efficacy using peritoneal exudate cells (Nash & Holle, 1973) from four sensitized guinea-pigs described previously. Antigen concentrations of 5 μg, 50 μg, 100 μg, 500 μg and 1000 μg (dry weight) per ml were used and optimal concentrations for detecting migration inhibition were between 20–100 μg/ml. Within this range there was no significant toxicity against human leucocytes. In work with eighteen infected and seven control non-infected subjects on Goodenough Island, an antigen concentration of 50 μg dry weight/ml was used.

The diameters of migrating leucocyte tufts were measured in two directions at right angles to each other using a microscope stage graticule (Graticules Ltd., England) mounted under the migration plate. For each tuft an estimate of the mean radius^2 was obtained. The migration index (MI) was then expressed as:

$$\mathrm{MI} = \frac{\text{mean radius}^2 \text{ of migrating cells in the presence of } T.\ concentricum \text{ antigen}}{\text{mean radius}^2 \text{ of migrating cells in the absence of antigen}}$$

Antibody detection

The method used for antibody detection was counterimmunoelectrophoresis (Mackenzie *et al.*, 1980). Two concentrations of antigen (190 and 42·5 mg dry weight per ml) were assessed against sera from fifty-two infected patients and non-infected controls from Goodenough Island.

RESULTS

Skin tests

A total of sixty-eight patients were skin tested and returned from the 48 h reading. Forty-six per cent (32) and 52% (36) of the patients had negative or immediate type (ITH) skin reactions respectively. Twenty-two per cent (15) of the patients had weak delayed-type (DTH) skin reactions exceeding 3 mm in diameter, but only 9% (6) had DTH reactions over 5 mm in diameter. Of these, four patients showed both DTH and ITH. None of the control subjects had positive skin test reactions, but 3 (30%) and 1 (10%) of the patients with *T. rubrum* infections showed ITH or DTH respectively.

There were no significant differences between the proportions of patients in each skin test group who had chronic infections of over 10 years duration [ITH (78%), DTH (66%), negative (66%) ($P > 0{\cdot}5$)] or who had less than 30% of their surface area involved [ITH (22%), DTH (33%), negative 34% ($p > 0{\cdot}5$)]. The total numbers of patients in each category were fifty-two and twenty-one respectively.

Leucocyte migration inhibition

The results of the leucocyte migration inhibition tests are shown in Fig. 1. The mean migration indices (MI) for infected and control groups were 0·88 (±0·31) and 0·89 (±0·16) respectively. These values are not significantly different ($P > 0{\cdot}5$). However it was noted that patients with less than 30% body surface area involvement were significantly more likely to have MIs below (5/7), as opposed to above (2/11), a value of 0·7 ($P < 0{\cdot}5$, Fisher's Test).

Serological responses

Seventy-eight per cent of fifty-three patients whose serum was tested by counterimmunoelectrophoresis showed precipitating antibody to the cytoplasmic antigen of *T. concentricum*. However there was no correlation between a positive antibody response and duration of infection or surface area involved (see skin test results). Only one control subject had antibody detected at the highest concentration of antigen (190 mg/ml).

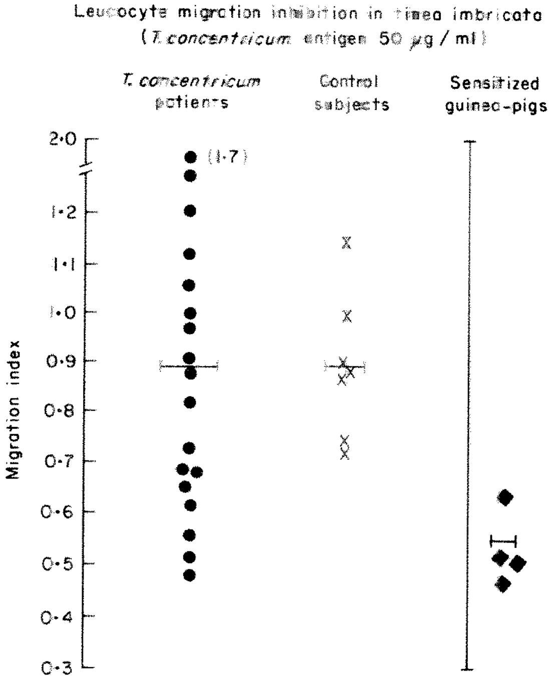

FIGURE 1. *In vitro* leucocyte migration inhibition responses in patients with tinea imbricata. The MIs obtained using peritoneal macrophages from sensitized guinea-pigs are shown for comparison.

DISCUSSION

We have shown that a significant proportion (52%) of patients with tinea imbricata showed immediate-type hypersensitivity (ITH) to *T. concentricum* antigen. A similar number (46%) were unreactive, whereas only 9% had significant delayed-type hypersensitivity (DTH) to the organism. This was similar to the results of a study using a commercial preparation of non-specific trichophytin (Hollister–Stier, U.S.A.) in East Sepik province where 76% (28) and 4% (1) of individuals tested showed ITH or DTH respectively (Maclennan, 1972). Negative intradermal tests or ITH have also been reported in a high proportion of patients in temperate countries with persistent dermatophytosis usually caused by *T. rubrum* (Jones, Reinhardt & Rinaldi, 1974a; Hay, 1982). In the present study it was also shown that there was *in vitro* T-lymphocyte hyporeactivity to the *T. concentricum* antigen measured by the leucocyte migration inhibition assay, although there were significant inhibitory responses (MIs less than 0·7) in those with a smaller body surface area affected (less than 30%). Similar T-lymphocyte hyporeactivity has been shown in chronic *T. rubrum* infections (Hanifin, Ray & Lobitz, 1974; Hay & Brostoff, 1977). Conversely, a high proportion of patients (78%) had detectable antibody to *T. concentricum*. This is an unusually high response rate for a dermatophyte infection where commonly less than 50% of those affected show antibody responses except in certain infections such as favus, caused by *T. schoenleinii* (Grappel, Blank & Bishop, 1972).

Much of the work on the immune responses in chronic dermatophytosis, cited above, has been carried out in countries where there are two major differences in the course and pattern of infections from those studied here. First, the commonest cause of persistent infection is *T.*

rubrum and second, the majority of such infections involve peripheral sites such as the feet. Widespread infections affecting the greater part of the body surface area, as occurs regularly in *T. concentricum* infections, are rarely seen. It has been shown that the site of infection may affect both skin test (Kaaman, 1978) and lymphocyte transformation (Hay & Shennan, 1982) responses with palmar/plantar infections being associated with weaker T-lymphocyte responses. However, in our present study areas with thick keratin, including palms and soles or scalp hairs, were not involved and it is likely that diminished responsiveness related to the site of infection would be minimized. The extensive nature of the infection may explain the high prevalence of detectable antibody. The identity of the infecting organism may also affect T-lymphocyte responses, with *T. mentagrophytes*, for instance, being more frequently associated with significant T-lymphocyte stimulation than *T. rubrum* (Hay & Shennan, 1982), and *T. concentricum* may be similar to the latter in this respect.

In chronically infected patients endogenous mediators may be responsible for negative skin tests and poor lymphocyte transformation responses (Jones, Reinhardt & Rinaldi, 1974a). The association between ITH and chronic dermatophytosis is well recognized although its significance is not clearly understood. However, histamine released following the interaction between specific antigen and IgE-bearing mast cells has been shown to inhibit T-lymphocyte transformation responses *in vitro* (Brostoff, Pack & Lydyard, 1980). The presence of a similar mechanism in infected patients might explain the persistence of infection, assuming that T lymphocytes are an essential part of the host response in dermatophytosis (Jones, Reinhardt & Rinaldi, 1974b). Alternatively the ITH may be a manifestation of uncontrolled IgE production due to a defect in the T-lymphocyte population which regulates its synthesis (Tada & Okumura, 1971). Peripheral blood eosinophilia, often associated with raised IgE levels, has been cited as a feature of patients with tinea imbricata (Maclennan & O'Keefe, 1975). Alternative explanations for T-lymphocyte hyporeactivity include the presence of blocking antibody, enhanced T suppressor-cell activity and disruption of lymphocyte recirculation (Bullock, 1976).

Whatever the reason for the apparent failure of T-lymphocyte responses, it is likely that this is responsible, in part, for persistence of tinea imbricata and its high relapse rate after treatment. Genetic predisposition (Serjeantson & Lawrence, 1977) and environmental phenomena such as humidity may also contribute. However no consistent clinical or biochemical underlying factors have been demonstrated in infected versus control populations, although the presence of hepatomegaly, low serum cholesterol levels and a high height/weight ratio have been cited as possible correlates of susceptibility in certain groups (Vines, 1970).

Tinea imbricata is not a common disease. However it may prove to be a valuable model for the study of cutaneous defence. There are few comparable infections in which such large areas of skin are chronically affected by such a large antigen load.

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Recent upper respiratory tract infection and pityriasis rosea: a case-control study of 249 matched pairs

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SUMMARY

A sample of 249 patients was drawn from an original population of 939 patients with pityriasis rosea identified in an earlier population-based study of pityriasis rosea in Rochester, Minnesota. A control was selected for each case, matched for age, sex, and year of diagnosis. Comparison was made with regard to recent immunization, history of atopy, and recent infection. A statistically significant number of patients had experienced recent upper respiratory tract infection ($P < 0{\cdot}005$). The association between recent infection and pityriasis rosea is discussed.

Pityriasis rosea is an inflammatory dermatosis of unknown aetiology with an acute self-limited course. In their review of the literature, Björnberg & Hellgren (1962) listed the conditions that could be precipitating factors, including infections, pregnancy, medications, seborrhoeic dermatitis, mental stress, garment contact, eczema, and earlier local skin lesions. Various reports have suggested possible links between pityriasis rosea and bacterial (Björnberg & Hellgren, 1962), viral (Karzon *et al.*, 1962; Raskin, 1968; Metz, 1977; Aoshima, Komura & Ofuji, 1981), or mycoplasmal (Grayston *et al.*, 1965; Feizi *et al.*, 1967) infections; others failed to implicate any infective agent (Wright & Francis, 1961; Garcia E Silva & Gardner, 1968; Hudson, Adelman & Lewis, 1981). In a case-control study, a higher incidence of asthma and eczema was found in relatives of patients with pityriasis rosea (Björnberg & Hellgren, 1962); this was interpreted by Burch & Rowell (1970) as representing the pleiotropic effect of one or more predisposing genes. They suggested that certain infective agents can precipitate an attack of pityriasis rosea in susceptible persons.

In our earlier population-based study of pityriasis rosea in Rochester, Minnesota (Chuang *et al.*, 1982), we reported a noticeable proportion of patients with recent or concurrent infection (21%) or a personal history of asthma, hay fever, or atopic dermatitis (16%). To evaluate the

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significance of these findings, we conducted a case-control study for 249 patients drawn from the original pool of 939 patients with pityriasis rosea.

METHODS

Selection of study population

The details of the original 939 patients identified in the earlier study of pityriasis rosea are reported elsewhere (Chuang *et al.*, 1982). Only 747 Mayo Clinic records of Rochester residents were used for the present study and of these, every third patient was selected as a case. Table 1 shows that the mean ages, sex ratios, mean lengths of follow-up, seasons of diagnosis, and incidences of atopy and recent infection of the 249 selected cases were similar to those of the original 939 patients.

Selection of control population

Utilizing the data from the centralized medical record index housed at the Mayo Clinic, one can select controls through the medical registration lists, which in any 3-year period constitute 90–95% of the total Rochester population. Selection of controls from such a pool of patients (who were seen for anything from minor problems such as refractions, pre-employment examinations, and the like, to the occasional more severe problems such as cancer) eliminates the bias common to the use of specialty clinic or hospital controls. A total of 249 controls were matched with cases by age, sex, and year of diagnosis; the person with the closest registration number was chosen, since the registration numbers are assigned in sequence and the duration of documented medical care was similar. Similar seasonal patterns of medical care usage existed for both case and control groups, e.g. 58% of controls and 57% of cases were seen in the colder months (October and March).

Conditions selected for comparison

Three categories of conditions were considered: (1) an immunization in the period 1 month before the visit for pityriasis rosea (case) or other problem (control); (2) a history of atopy, defined as asthma, hay fever, or atopic dermatitis; and (3) an infection experienced in the 3 months before the visit. Included in this category were upper respiratory tract infections

TABLE 1. Selected features of 249 cases compared with the original 939 patients (Chuang *et al.*, 1982) with pityriasis rosea in Rochester, Minnesota

	Selected 249 cases	Original 939 cases
Age, mean (yr)	23·2	22·7
Sex ratio (M:F)	1:1·9	1:1·8
Diagnosed in colder months (%)	57·4	59·6
Follow-up (yr)*	14·9	14·4
Atopy (%)	14	16
Recent infection (%)	12	14

* Follow-up period is calculated from the first visit to a clinic for any medical reason to the last visit

(streptococcal, viral, or unspecified), dental infection, otitis media/externa, urinary tract infection, herpes simplex, herpes zoster, vulvovaginal candidosis, sexually transmitted disease, deep skin infection, pneumonia, viral gastroenteritis, and miscellaneous infections. Diagnoses of the above conditions were made by physicians and were recorded at visits prior to that for the index disease (case or control). A patient's own recall of such an infection was not counted as a legitimate episode of infection. For the diagnosis of upper respiratory tract infection, culture for suspected infectious agents was pursued in four-fifths of the cases and controls. 'Concurrent' infection was not considered in this study in view of the uncertainty of the temporal relationship between infection and pityriasis rosea (case) or other problems (control).

Statistical analysis

Two-sided sign tests for comparison of the differences in proportions were employed. Odds ratios for matched pairs were estimated, and 95% confidence intervals are given.

RESULTS

Table 2 shows selected results and P values of the matched-pair comparisons. Most of these, including those not shown in the table, did not reveal significant differences. Here we cite a few points worthy of mention.

Recent immunization. Only 4% of cases and 5% of controls received any kind of immunization in the month before the diagnostic visit. No significant difference was noted in the proportion immunized.

Atopy. Fourteen percent of cases and 12% of controls had histories of atopy. The difference in proportion was due primarily to asthma, which occurred more often in the cases (7%) than in the controls (4%); however, this difference is not statistically significant (P = 0·29).

Recent infection. Twelve percent of cases and 6% of controls had experienced infection within 3 months of diagnosis (P = 0·014). This difference was due primarily to upper respiratory tract infection (8% of cases and 2% of controls, P = 0·004), in nearly half of which a positive throat culture was obtained.

TABLE 2. Selected statistical results of 249 matched-pair comparisons with respect to pityriasis rosea in Rochester, Minnesota

	Case + Control −	Case − Control +	P value*	Odds ratio	95% confidence interval
Recent immunization	8	10	0·81	0·80	0·27–2·25
Atopy:	30	24	0·50	1·25	0·71–2·23
Asthma	14	8	0·29	1·75	0·69–4·81
Recent infection:	30	13	*0·014*	2·31	1·17–4·82
URI†	18	4	*0·004*	4·50	1·48–18·27

* Two-sided sign test for matched pairs; statistically significant P values are italicized.

† Upper respiratory tract infection.

DISCUSSION

In spite of the suggestion that pityriasis rosea might be an autoimmune disease affecting genetically susceptible persons with atopy (Burch & Rowell, 1970), such an association could not be substantiated by this study. However, the present case-control study does suggest that a recent upper respiratory tract infection is strongly associated with the subsequent development of pityriasis rosea in a small proportion of patients. The infectious aetiology of pityriasis rosea has been favoured in some investigations (Björnberg & Hellgren, 1962; Karzon *et al.*, 1962; Grayston *et al.*, 1965; Feizi *et al.*, 1967; Raskin, 1968; Metz, 1977; Aoshima *et al.*, 1981), although the findings are far from conclusive (Wright & Francis, 1961; Garcia e Silva & Gardner, 1968; Hudson *et al.*, 1981). A recent report stated that virus-like particles were observed by electron microscopy in the herald patch of pityriasis rosea of two patients (Aoshima *et al.*, 1981). The viruses were suggested to be RNA-containing, belonging to alpha-virus of the togavirus or arenavirus. Other reports have linked pityriasis rosea to picornavirus-like particles (Raskin, 1968; Metz, 1977), ECHO 6 virus (Karzon *et al.*, 1962), or to mycoplasma (Grayston *et al.*, 1965; Feizi *et al.*, 1967). A study for T and B lymphocytes in pityriasis rosea raised speculation that 'viral agents' may be responsible for the transient increase in peripheral IgE/IgM-bearing cells (Kermani-Arab, Roberts & Leslie, 1978). In Björnberg & Hellgren's (1962) investigation, previous cutaneous infections, which included furuncles, carbuncles, and impetigo contagiosa, were found more often in patients with pityriasis rosea ($P < 0{\cdot}01$). In the present series, recent deep skin infection occurred in only 2% of cases and 1% of controls, a difference that is not statistically significant ($P = 0{\cdot}25$).

Additional support for the infectious aetiology of pityriasis rosea has come from several alleged 'epidemics' reported by investigators (Percival, 1932; Crissey, 1956; Marshall, 1956; Abercrombie, 1968) and from some other interesting observations (White, 1973; McPherson, McPherson & Ryan, 1980; Bosc, 1981; Messenger *et al.*, 1982). The results of a British questionnaire survey suggested that an infectious aetiology may be responsible for the threefold to fourfold increase in incidence of pityriasis rosea which affected dermatologists compared with ear, nose and throat surgeons and pre-specialization dermatologists (McPherson *et al.*, 1980). This difference, after standardization for age, is highly significant ($P < 0{\cdot}001$). A significant degree ($P < 0{\cdot}005$) of space-time clustering of cases was found in another British study of 126 patients with pityriasis rosea seen over 2 years (Messenger *et al.*, 1982). These findings support the hypothesis that pityriasis rosea is caused by an infective agent (Messenger *et al.*, 1982). Concurrence of cases in the same household (Björnberg & Hellgren, 1962; Abercrombie, 1968; Chuang *et al.*, 1982) or through alleged close contact (Abercrombie, 1968; White, 1973; Bosc, 1981) further favours the hypothesis of an infectious agent. The seasonal pattern of pityriasis rosea, with more frequent occurrence in colder months (Crissey, 1956; Abercrombie, 1968; Messenger *et al.*, 1982; Chuang *et al.*, 1982), is compatible with the pattern of increased incidence of respiratory infection during the fall and winter.

Since the recent infections cited in this study were only those diagnosed by physicians at clinic visits, the 12% in the present series should be seen as minimal, for an unknown proportion of persons with mild, transient, or asymptomatic infections do not seek medical assistance. Although the possibility that pityriasis rosea has multiple causes or a complicated aetiology cannot be ruled out, the role of recent infection, particularly upper respiratory tract infection, in the development of pityriasis rosea is worth noting.

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Pharmacology and Treatment

Hydroxychloroquine versus phlebotomy in the treatment of porphyria cutanea tarda

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SUMMARY

Hydroxychloroquine and phlebotomy were compared in the treatment of porphyria cutanea tarda (PCT). Thirty patients received hydroxychloroquine (200 mg twice weekly) for 1 year and thirty-one underwent twice-monthly phlebotomies of 400 ml whole blood each, also for 1 year. Clinical signs of disease improved equally in both groups. At the end of the year, urinary porphyrin excretion had significantly improved in twenty-two out of thirty hydroxychloroquine-treated subjects, but in only eight out of the thirty-one patients who received phlebotomy. Liver histology showed significant regression of steatosis and siderosis in both groups compared with the pretrial biopsy, but the activity of liver disease, as judged by the extent of necrosis, inflammation and fibrosis, worsened in twelve hydroxychloroquine and in seven phlebotomy-treated patients. It is concluded that hydroxychloroquine is more effective than phlebotomy in decreasing porphyrin production. However, further work is needed to assess whether long-term hydroxychloroquine treatment favours the progression of the chronic liver disease associated with PCT.

Porphyria cutanea tarda (PCT) is a syndrome characterized by skin fragility, photosensitivity and a distinctive pattern of urinary porphyrin overexcretion, which consists predominantly of 8- and 7-carboxylporphyrins and lesser amounts of 6-, 5- and 4-carboxylporphyrins (Hynes, 1980). Most of the clinical and biochemical features of PCT seem to depend on the deficient activity of the enzyme uroporphyrinogen decarboxylase (URO-D). PCT can exist in two forms: familial, when the enzyme is defective both in erythrocytes and liver cells, and sporadic characterized by deficient activity only in the liver (Smith & De Matteis, 1980). Whatever the type of underlying enzymatic defect, repeated phlebotomies (Lundvall, Weinfeld & Lundin,

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1970; Walsh *et al.*, 1970; Grossman *et al.*, 1979; Di Padova *et al.*, 1983) or hydroxychloroquine (Vogler, Galambos & Olanski, 1970; Chlumska, Chlumsky & Malina, 1980; Malkinson & Levitt, 1980) have salutary effects on the clinical and biochemical hallmarks of PCT.

No randomized clinical trial has previously been performed to compare the effectiveness of these two treatments on the urinary porphyrin profile and liver morphology. Therefore, we decided to evaluate over a 1-year period the clinical and biochemical course of PCT and the histological evolution of the concomitant liver disease in a series of sixty-two patients treated with hydroxychloroquine or phlebotomy.

METHODS

The study group comprised sixty-two male patients in whom PCT had been diagnosed on the basis of typical skin features and anomalous porphyrin pattern (Hynes, 1980). None had a family history of porphyria and none had been previously treated for PCT. All patients admitted having consumed alcohol in excess of 80 g daily for the past year.

An initial assessment was made of drinking history, physical conditions and various haematological and biochemical tests. Particular note was made of liver function tests and urinary porphyrins and a liver biopsy was performed by Tru-Cut needle (Travenol, Italy). The patients were then randomly assigned to two equal groups of thirty-one by the study monitor (L.M.) who did not know the results of liver function tests and urinary porphyrin levels. The first group (mean age 50 ± 7 years, range 33–70 years) received hydroxychloroquine (Plaquenil®) at a dose of 200 mg twice weekly for 12 months, and the second group (mean age 48 ± 8 years, range 29–67 years) underwent a 1-year course of phlebotomy. At each twice-monthly venesection 400 ml of whole blood was removed. This schedule was chosen since our previous experience (Di Padova *et al.*, 1983) indicated that twice-monthly phlebotomies are better tolerated than weekly venesections by patients with chronic liver disease at risk of developing hypovolaemia and hypoalbuminaemia. Blood haemoglobin concentration was always maintained above 10 g/dl. Patients in both groups were advised to stop alcohol intake and their compliance was assessed by personal interview, and also by serum γ-glutamyltranspeptidase and degree of liver steatosis (Orrego *et al.*, 1979; Chick, Kreitman & Plant, 1981). Patients were seen every 4 months at which time full clinical biochemical reassessment was performed. After 12 months of treatment all patients were readmitted to hospital for liver biopsy.

Analytical methods

Complete blood count and liver function tests (bilirubin, transaminases, γ-glutamyltranspeptidase, serum protein electrophoresis, etc.) were performed by routine methods. Hepatitis B surface antigen and antibody were determined by radioimmunoassay (Ausria II-125 and Ausab, Abbott Laboratories, Chicago, IL). Serum iron and total iron binding capacity were measured as previously described (Cook, 1970; International Committee for Standardization in Haematology, 1971). Urine specimens were tested for porphobilinogen according to Doss (1971); total porphyrins were determined by ion exchange chromatography as described by Doss & Schmidt (1971). Quantification of uro- and coproporphyrins and intermediate compounds with seven, six and five carboxyl groups (hepta-, hexa- and penta-carboxylporphyrins) was performed by high pressure liquid chromatography (HPLC) according to Gray, Lim & Nicholson (1977).

Urinary porphyrins were absorbed on talc, esterified with sulphuric acid in methanol and extracted with chloroform (Doss, 1970). Porphyrin methylesters were then fractionated by HPLC with a chromatograph including a Model 6000 A solvent delivery system, Model 660 solvent programmer, Model U6K injector and Model 450 variable wavelength detector set at 404 nm (all from Waters Associated, Milford, MA). A stainless steel μ-Porasil column (30 cm × 4 mm) was used with the mobile phase consisting of n-heptane/methylacetate (3:2, v/v). A chromatographic marker porphyrin esters kit (Porphyrin Products, Logan, UT), containing 8, 7, 6, 5, 4 carboxylporphyrins as their methyl esters was used as standard.

Urinary porphyrin methylester profiles produced by quantitative HPLC were classified before and after the 1-year treatment following the scheme proposed by Doss (1980). This scheme classifies overt PCT as type D and subdivides the subclinical and latent stages of the disease into types A, B and C, according to the increasing severity of the alterations of urinary porphyrin profiles. In this classification, type A is characterized by an increase in the percentage of excretion of uroporphyrins with a ratio of copro- to uroporphyrins greater than 1·0, type B by the ratio of copro- to uroporphyrins less than 1·0 and type C by a massive increase in the excretion of heptacarboxyl-porphyrins too. The distribution pattern of porphyrins in type C is similar to that in stage D which represents the clinically overt form of the disease.

Liver specimens obtained at the beginning and end of the clinical trial were fixed in 10% formalin and processed for light microscopy using the routine staining procedures plus iron stain as described by Scheuer (1980). Overall assessment of histological improvement or worsening in each patient was based on evaluation of the extent of necrosis, inflammation and fibrosis. All biopsies were classified according to the criteria devised by Bianchi *et al.* (1977).

According to other authors (Bruguera, Bordas & Rodes, 1977; Goldberg *et al.*, 1977; Cortes *et al.*, 1980) either chronic persistent hepatitis or chronic active hepatitis was diagnosed in these alcoholic patients when the characteristics of the inflammatory infiltrates and fibrosis differed from those of alcoholic hepatitis (Baptista *et al.*, 1981). Each liver specimen was graded for fat on a scale from 0 to +4, according to five levels of severity, as reported by Abdi, Millan & Mezey (1979). Thus fatty infiltration of the liver was graded as follows: 0, none; +1, none to 25%; +2, 25% to 50%; +3, 50% to 75%; and +4, more than 75%.

The amount of parenchymal iron was assessed according to Scheuer (1980): grade 0 indicated absence of siderosis, while grades +1 to +4 represented increasing degrees of iron deposition.

The results are expressed as mean ± s.e. Statistical analysis was by Student's t test for paired data; differences in means were calculated by Student's t test for unpaired data. Differences in porphyrin pattern distribution were tested by the χ^2 test.

RESULTS

One non-compliant patient was withdrawn from the trial so that sixty-one subjects completed the study, thirty in the hydroxychloroquine group and thirty-one in the phlebotomy group. All patients claimed to have reduced their alcohol intake (less than 25 g/day) during the year-long trial.

Both treatments were well tolerated and produced no clinical side-effects except for mild anaemia associated with repeated phlebotomies. Cutaneous symptoms showed a comparable improvement in both groups of patients at the eighth month of treatment, with regression of phototoxic vesicles, skin fragility and hyperpigmentation.

Initial liver function tests and other laboratory data were similar in the two treatment groups (Table I) and did not differ between groups at 4 and 8 months of therapy (data not reported), with the exception of serum glutamic oxalacetic transaminase and iron, which were lowered more in the phlebotomy group.

The same laboratory data at the end of the 12-month trial and the differences between

TABLE I. Changes from baseline of selected laboratory data in hydroxychloroquine- and phlebotomy-treated PCT patients

	Normal value	Baseline	After 1 year of treatment	Absolute change
Serum bilirubin	(0·30–1·00 mg/dl)			
Hydroxychloroquine		0·78 ± 0·05	0·66 ± 0·04	−0·12 ± 0·05
Phlebotomy		0·70 ± 0·06	0·62 ± 0·05	−0·11 ± 0·05
Serum glutamic-oxalacetic-transaminase	(0–20 mU/ml)			
Hydroxychloroquine		36 ± 4	43 ± 14	7 ± 13
Phlebotomy		34 ± 5	23 ± 2*	−12 ± 5
Serum glutamic-pyruvic-transaminase	(0–24 mU/ml)			
Hydroxychloroquine		33 ± 3	30 ± 4	−6 ± 3
Phlebotomy		28 ± 4	18 ± 2	−10 ± 4
Serum cholinesterase	(1900–3800 mU/ml)			
Hydroxychloroquine		2883 ± 163	3059 ± 183	121 ± 218
Phlebotomy		2584 ± 136	2433 ± 143	−118 ± 133
Serum γ-glutamyl-transpeptidase	(6–28 mU/ml)			
Hydroxychloroquine		107 ± 14	57 ± 9‡	−53 ± 13
Phlebotomy		99 ± 10	32 ± 5§	−69 ± 9
Serum total proteins	(5·5–8·0 g/dl)			
Hydroxychloroquine		7·5 ± 0·1	7·4 ± 0·1	−1·9 ± 1·7
Phlebotomy		7·5 ± 0·2	7·4 ± 0·1	−0·8 ± 1·4
Serum albumin	(50–60% of total proteins)			
Hydroxychloroquine		55·0 ± 0·8	57·8 ± 0·8	2·2 ± 0·7
Phlebotomy		51·4 ± 1·2	53·8 ± 1·1	5·5 ± 0·8
Serum γ-globulins	(13–23% of total proteins)			
Hydroxychloroquine		20·7 ± 0·7	18·8 ± 0·7	−0·2 ± 1·0
Phlebotomy		20·7 ± 1·1	22·5 ± 0·9	0·5 ± 0·7
Serum iron	(50–150 μg/dl)			
Hydroxychloroquine		204 ± 11	160 ± 8†	−24 ± 7
Phlebotomy		184 ± 10	87 ± 7§	−65 ± 10**
Serum iron binding capacity	(250–400 μg/dl)			
Hydroxychloroquine		344 ± 8	355 ± 7	24 ± 7
Phlebotomy		353 ± 9	415 ± 12†	65 ± 10††

Normal values in parentheses. Results are expressed as the mean ± S.E. * $P < 0{\cdot}05$ vs. baseline; † $P < 0{\cdot}01$ vs. baseline; ‡ $P < 0{\cdot}005$ vs. baseline; § $P < 0{\cdot}001$ vs. baseline; ** $P < 0{\cdot}01$ between absolute changes; †† $P < 0{\cdot}05$ between absolute changes.

absolute changes are also shown in Table I. Serum bilirubin, glutamic pyruvic transaminase, cholinesterase and protein electrophoresis did not change during the study. Serum glutamic oxalacetic transaminase at the end of the year was significantly lower than initial levels only in the phlebotomy group, whereas γ-glutamyltranspeptidase levels markedly decreased in both groups. Serum iron dropped maximally in phlebotomy-treated patients so that the absolute changes were significantly different from the hydroxychloroquine group though this group's final values were significantly lower than their initial ones.

After the 1-year treatment a marked reduction in the percentage saturation of transferrin was found in the phlebotomy group compared with the hydroxychloroquine group (20 ± 2% vs. 45 ± 3%, $P < 0{\cdot}005$).

Screening for urinary porphobilinogen was always negative. As shown in Fig. 1, urinary total

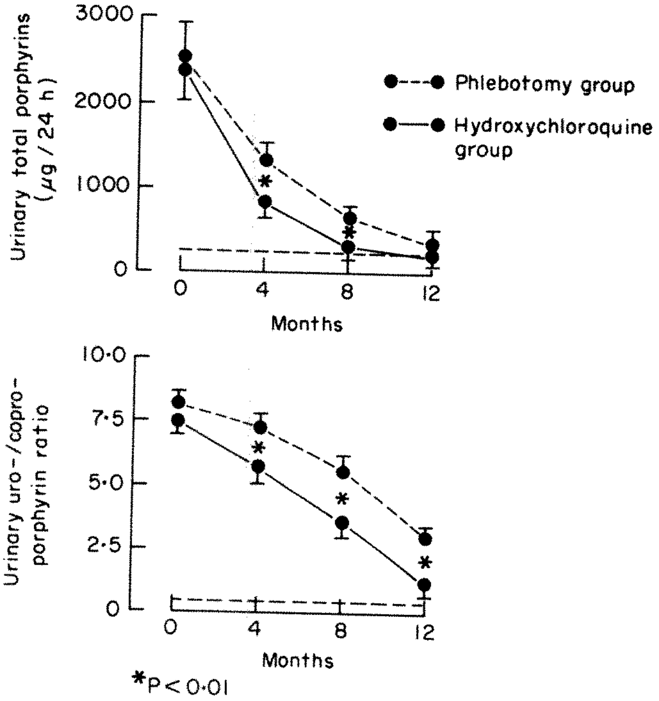

FIGURE 1. Effects of hydroxychloroquine and phlebotomy on urinary total porphyrins and uro-/copro-porphyrin ratio in patients with PCT. Each point represents the mean ± s.e. of thirty-patients for hydroxychloroquine and thirty-one cases for the phlebotomy group. Normal urinary total porphyrin excretion and uro-/copro-porphyrin ratio are less than 200 mcg/24 h and 0·25, respectively. Upper limits of normal values are shown by horizontal dashed lines. The asterisk indicates significant differences ($P < 0{\cdot}01$) between groups at the time considered.

porphyrins decreased in both treatment groups, but the drop was significantly greater after hydroxychloroquine at 4 and 8 months of therapy. In parallel, the uro-/coproporphyrin ratio, obtained from analysis of HPLC porphyrin profiles, was significantly lower in hydroxychloroquine than in the phlebotomy group at every test.

At baseline all patients exhibited a urinary porphyrin pattern classified as stage D PCT, characterized by a high prevalence of uro- and heptacarboxyl-porphyrins. At the end of the 1-year trial, the urinary porphyrin pattern had improved more in the hydroxychloroquine than in the phlebotomy group (Table 2). Intermediate porphyrins with five, six and seven carboxyl

TABLE 2. Distribution of urinary porphyrin patterns, classified according to Doss (1980), after 1 year of therapy with hydroxychloroquine or phlebotomy in patients with PCT

	Normal No. (%)	Type A No. (%)	Type B No. (%)	Type C No. (%)	Type D No. (%)
Hydroxychloroquine	22 (73)	3 (10)	1 (3)	4 (13)	— —
Phlebotomy	8 (25)	6 (19)	9 (29)	8 (25)	— —

No. = number of cases

TABLE 3. Liver biopsy diagnoses before and after 1 year of therapy with hydroxychloroquine or phlebotomy in patients with PCT

	MC	F	AH	CPH	CAH	C
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Hydroxychloroquine						
Baseline	— —	3 (10)	4 (13)	8 (26)	11 (36)	4 (13)
After 1 year of therapy	— —	1 (3)	2 (6)	9 (30)	10 (33)	8 (26)
Phlebotomy						
Baseline	— —	1 (3)	5 (16)	8 (25)	10 (32)	7 (22)
After 1 year of therapy	3 (9)	1 (3)	1 (3)	7 (22)	10 (32)	9 (29)

MC = Minimal Changes; F = Fibrosis; AH = Alcoholic Hepatitis; CPH = Chronic Persistent Hepatitis; CAH = Chronic Active Hepatitis; C = Cirrhosis. No. = number of cases.

groups disappeared in twenty-two of the patients receiving the drug, but only eight patients in the phlebotomy group achieved a normal pattern and normal total porphyrin excretion in urine (less than 200 mcg/24 h). The difference of porphyrin pattern distribution between the two groups was highly significant ($P < 0{\cdot}01$) by χ^2 test. The morphological data regarding liver biopsy findings are shown in Table 3. Follow-up of the histological data revealed that four hydroxychloroquine-treated and two phlebotomy-treated subjects worsened into cirrhosis. The activity of the liver disease, judged by the extent of necrosis inflammation and fibrosis, worsened compared with pretrial conditions in twelve hydroxychloroquine-treated and seven phlebotomy-treated patients. The degree of fatty liver degeneration significantly improved in both treatment groups, from $1{\cdot}9 \pm 0{\cdot}2$ to $1{\cdot}0 \pm 0{\cdot}1$ ($P < 0{\cdot}001$) after hydroxychloroquine and from $2{\cdot}1 \pm 0{\cdot}2$ to $0{\cdot}9 \pm 0{\cdot}1$ ($P < 0{\cdot}001$) after phlebotomy. Siderosis was also reduced in both groups, from $1{\cdot}8 \pm 0{\cdot}2$ to $1{\cdot}3 \pm 0{\cdot}1$ ($P < 0{\cdot}05$) after hydroxychloroquine, and from $2{\cdot}0 \pm 0{\cdot}1$ to $1{\cdot}0 \pm 0{\cdot}1$ ($P < 0{\cdot}001$) after phlebotomy. The absolute change evaluated by Student's t test was significantly higher ($P < 0{\cdot}05$) in the phlebotomy group than in the hydroxychloroquine group ($-0{\cdot}94 \pm 0{\cdot}13$ vs. $-0{\cdot}50 \pm 0{\cdot}16$).

DISCUSSION

The present study, comparing hydroxychloroquine and phlebotomy in the treatment of PCT, confirmed previous reports of the effectiveness of both these therapies. At the end of the 1-year trial, twice-monthly phlebotomies induced a degree of biochemical remission of PCT (total 24-h urine porphyrins less than 400 mcg) comparable to that reported by Grossman *et al.* (1979) who employed a more exhaustive protocol of weekly venesections. According to our data, some differences were recorded in the response of urinary porphyrins to the two therapies. The reduction of total porphyrins and the uro-/coproporphyrin ratio was faster in the hydroxychloroquine than in the phlebotomy group. Determination of the porphyrin profile offers a sensitive tool for assessing the course of the disease and the subsequent therapeutic approach. Thus in the twenty-four patients of the phlebotomy group with anomalous patterns at the end of the clinical trial, therapy was continued up to 18 months at which time fifteen subjects had normal urinary total porphyrin excretion and profile. The delay in therapeutic effect can be tentatively related to the indirect action of venesection which probably depends on the removal of iron, one putative factor in the development of PCT (Kushner, 1982). On the other hand, the prompt beneficial effect of hydroxychloroquine may simply be attributed to its specific ability to mobilize and remove lysosomal stores of porphyrins (Chlumska *et al.*, 1980; Malkinson & Levitt, 1980; Riches, Morris & Stanworth, 1981; Harder, Pakalapati & Debuch, 1981). However, more complex biological interactions of the drug with cellular structures such as mitochondria, which are a site of porphyrin biosynthesis (Kowertz, 1976), cannot be ruled out.

With regard to the therapeutic value of alcohol abstinence, we have previously demonstrated that this measure by itself fails to produce remission of PCT (Di Padova *et al.*, 1983) although it certainly has an adjunctive role in maintaining remission of the disease.

The criteria of improvement of PCT used in this study were clinical, biochemical and morphological. From this point of view, the liver disease, judged by the extent and activity of necrosis, inflammatory infiltrates and fibrosis, as well as by serum GOT, seemed more severe in the hydroxychloroquine than in the phlebotomy group. Six patients (four in the hydroxychloroquine and two in the phlebotomy group) worsened into cirrhosis by the end of the trial. However, interpretation of these data is made difficult by the wide histological variations between patients with PCT. These alcoholic patients exhibited a variety of pretrial diagnoses which often contrasted with the diagnosis of alcoholic liver disease (Baptista *et al.*, 1981). The apparent discrepancy can be tentatively explained by the concomitance of alternative aetiologies in the same subject. Thus, ten out of sixty-two patients gave a positive test for the hepatitis B surface antigen or antibody, six in the hydroxychloroquine and four in the phlebotomy group.

Steatosis and siderosis showed significant regression in this series of patients; most likely this is related to the effects of alcohol abstinence plus specific therapy. Abstinence from alcohol may also have contributed to the low final serum iron values in the hydroxychloroquine treated group.

In conclusion, we found that hydroxychloroquine is more effective than phlebotomy at removing porphyrins. The study also showed that the liver disease associated with PCT improved less in the hydroxychloroquine than in the phlebotomy group, but this cannot be certainly attributed to hydroxychloroquine hepatotoxicity, since other events such as the degree of alcohol abstinence or infection with hepatitis B virus may be important. However, further investigations are needed to elucidate the safety of long-term, low-dose hydroxychloroquine administration in patients with PCT and concomitant chronic liver diseases.

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Trigeminal trophic syndrome: a successful treatment with transcutaneous electrical stimulation

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SUMMARY

A patient with chronic ulceration of the face due to trigeminal nerve transection was successfully treated with transcutaneous electrical stimulation (TES). This treatment leads to enhancement of local blood supply with consequent improved wound healing.

Neurotrophic ulcers of the face may develop in some patients when a large proportion of fibres of the fifth cranial nerve are interrupted through infarction (Freeman, 1966), through degeneration (Spillane & Urich, 1976), or by tumour growth or artificial destruction (Morello, Bianchi & Migliavacca, 1971; Henderson, 1967). To treat trigeminal neuralgia, surgical section or alcohol injection of the semilunar ganglion of Gasser is often carried out. We report here a new treatment for this chronic and indolent condition.

CASE REPORT

A 56-year-old female was admitted to our department in January 1980. She had a 10-year history of severe trigeminal neuralgia which did not respond to drug therapy. In 1978 she had undergone section of the trigeminal nerve root by the method of Dandy. Two months after this operation small ulcers appeared, initially at the right side of her nose, later elsewhere on the involved side of the face. She also complained of paraesthesiae in the right nasal mucosa.

Examination revealed multiple, superficial, sharply delineated ulcers on the right half of the face (Fig. 1). Sensation to pressure, heat and cold was absent. The corneal reflex was negative but there was no evidence of exposure keratitis. A penicillin-resistant *Staphylococcus aureus* was cultured from one ulcer. Using infrared thermography it appeared that the right half of the face was cooler than the normal left half, except at sites of ulceration, where the temperature was increased. The sweat test (iodine starch) demonstrated anhidrosis of the right half of the face.

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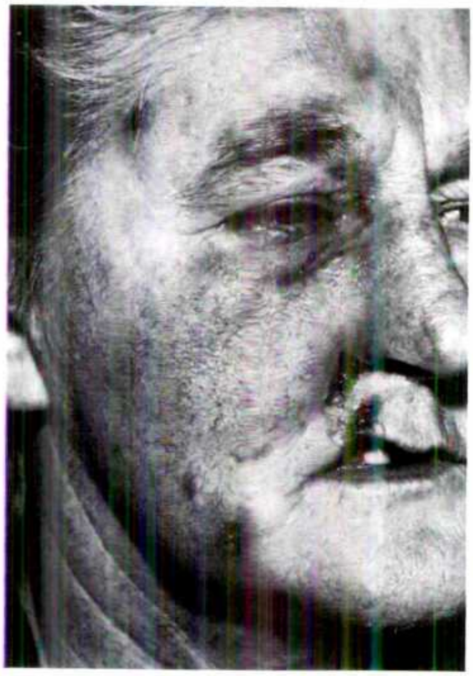

FIGURE 1. Neurotrophic ulcers of the face, before transcutaneous electrical stimulation.

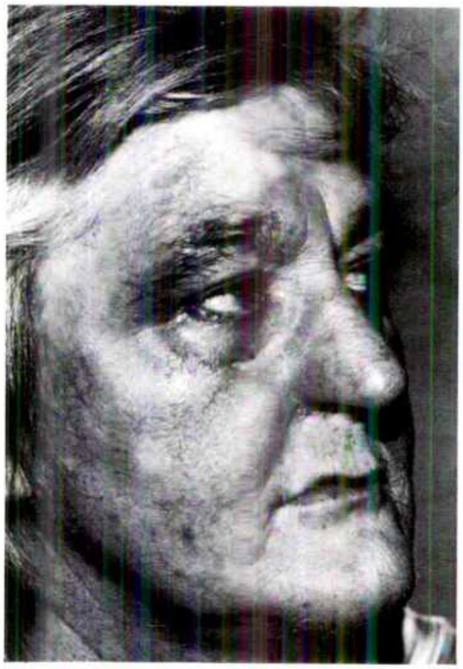

FIGURE 2. After transcutaneous electrical stimulation.

The patient was treated with systemic antibiotics without success. Some improvement occurred after covering the ulcers with non-adherent bandages but no definite healing process was observed.

A second admission was required when the patient developed cellulitis of the right orbicular region. The extent of the ulceration had not changed.

Based on the results of current research in this department concerning enhancement of local blood supply, we treated her with transcutaneous electrical stimulation (TES) using a Bio-Medical Research (BMR) P_8 unit professional (Westerhof *et al.*, unpublished). Flexible carbon rubber electrode pads (diameter 10 cm) were moistened with physiological saline and positioned on the lesional side of the face. The leads from the pads were connected to the BMR unit which was then adjusted to the following settings: frequency 120 Hz, pulse width 250 μsec, pulse train envelope 0·5 sec and pulse train interval 0·5 sec. The intensity was increased to obtain more vigorous contractions. This treatment was applied three times a day for periods of 30 min. The ulcers healed completely after 6 weeks (Fig. 2). Without further treatment the ulcers unfortunately recurred after some weeks but during subsequent treatment with TES, healing of the ulcers was again quickly accomplished.

DISCUSSION

Ulceration of the anaesthetic side of the face may occur after destruction of the divisions of the trigeminal nerve. Psychological problems are frequently associated with this trigeminal trophic syndrome (Bauer, 1966; Jackson, 1964; Miglets, 1968; Feder & Acquarelli, 1965) and are thought to predispose the patient to self-destructive behaviour. Although trauma in the anaesthetic area is often self-inflicted, it is noteworthy that the wounds ulcerate in an area of the body which is known for its quick healing. It may be that in destroying the Gasserian ganglion not only sensory qualities such as heat, cold, pain and proprioception are lost, but also autonomic vasomotor functions. Sympathetic fibres arising from the neighbouring internal carotid plexus which links with the trigeminal nerve are also interrupted. This causes a relative inbalance in the regulation of vasomotor activity in the area supplied by the trigeminal nerve.

This low sympathetic tone was demonstrated in our patient by infrared thermography and by the sweat test. The constant cooling of the skin due to vasodilation and slower venous return is an unfavourable condition for metabolic processes relating to wound healing. It may instead give rise to ulceration after traumatisation. Our patient suffered from severe ulceration in the right side of the face for 2 years and did not respond to wound treatment or antibiotic therapy. The enhancement of the local blood supply by TES appears to be of value in curing this intractable and sometimes mutilating condition.

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Case Reports

Urticarial vasculitis with shock, leukopenia and thrombocytopenia, possibly due to anaphylatoxin release

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SUMMARY

A case of hypocomplementaemic urticarial vasculitis, associated with shock, thrombocytopenia and leukopenia, is reported. We discuss the possibility that a single mechanism involving the anaphylatoxins C3a and C5a may explain the shock-like condition, the urticarial vasculitis and the blood abnormalities.

In cutaneous allergic vasculitis immune complexes activate complement (either by the classical or alternate pathways) and release its active fractions, including chemotactic fractions (C5a, C567) which may induce purpuric infiltrated lesions (Sams *et al.*, 1976).

We report the case of a patient who simultaneously demonstrated hypocomplementaemia, cytopenia and 'shock' with urticaria. Later, the urticarial lesions became purpuric. This suggested the diagnosis of hypocomplementaemic vasculitis and it seems likely that anaphylatoxins (C3a, C5a) could be responsible for the cytopenia and anaphylactic-like manifestations which preceded the classical type III phenomena (Coombs & Gell, 1975).

Hypocomplementaemic vasculitis is characterized by strikingly low C1q levels (Sams *et al.*, 1976; Craddock *et al.*, 1977; Penttinen, Vaheri & Myllyla, 1971), with urticaria-like lesions, arthralgia and histological findings of leukocytoclastic vasculitis (Agnello, 1978; Zeiss *et al.*, 1980). The unusual association of shock and thrombo-leukopenia supports the hypothesis that activated complement components (C3a–C5a) could be responsible for the anaphylactic-like reaction seen in our patient.

CASE REPORT

A 57-year-old male was admitted with a flare-up of urticaria. He was febrile (40°C) and in shock,

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with 'pinched' nose, marbled skin, systolic blood pressure of 70 mm Hg, pulse 120/min, oliguria and renal failure. Urticarial lesions were present over his whole body. He gave a history of abdominal pains and arthralgia of knees and ankles.

This acute episode occurred one week after oral antibiotic therapy with erythromycin. In the past 20 years the patient had suffered four episodes of acute polyarthritis (once with synovitis) and several episodes of common urticaria. From 1978, episodes of acute giant urticaria, often preceded by laryngeal discomfort, were complicated by collapse. Emergency laboratory investigations showed: haematocrit 50%; WBC 2600/mm^3 with 10% neutrophils; platelets 50,000/mm^3 (plasma clotting studies ruled out intra-vascular coagulation and fibrinolysis, with fibrinogen 4 g/l, FDP 10 μg, ethanol-test negative, and recalcification and prothrombin times normal); erythrocyte sedimentation rate 50 mm in the first hour; urea 17·5 mmol/l and creatine 150 mmol/l with no albuminuria; Addis count normal; Na/K < 2 in urine. Normal level of IgE.

The shock syndrome and the fever responded very well, without antibiotics, to injections of hydrocortisone hemisuccinate (400 mg).

Progress

Within 3 days, the uriticaria-like lesions spread over the body and became entirely purpuric and infiltrated. They faded within 2 weeks.

Laboratory data during the first week showed a persistent leukopenia and thrombocytopenia. Immunological investigations during the acute phase showed the presence of increased levels of circulating immune complexes by the C_{1q} binding test, viz. 87·7% (normal 46·4 ± 15·6), and by PEG precipitation, viz. 1·67 (normal 0·44 ± 0·17). The size of the immune complexes was not determined. Complement determination showed CH50 < 16 U 50%/ml (normal 35–65), C_{1q} 200 mg/100 ml (normal 1500–2000), C3 20 mg/100 ml (normal 60–125) and C4 500 μg/ml (normal 200–800). C5 was not evaluated.

The IgE level was normal. Tests for antinuclear and anti-DNA antibodies were negative.

Two days later, C3 was still low (30 mg/100 ml), but C4 decreased (112 μg/ml). Four days later, the CH50 level was raised to normal (61·7 U 50%/ml) with a reduced value for C3 (37 mg/100 ml) and C4 (150 μg/ml). The C5 level was normal. There were no antinuclear antibodies, cryoglobulins, cryofibrinogen, HBs-antigen or antibody demonstrated. The IgE level increased dramatically to 2265 μg/l (normal < 350 μg/l). RAST tests demonstrated a polysensitization.

Biopsy of a purpuric skin lesion (performed on the fourth day of hospitalization) showed endothelial swelling, fibrinoid necrosis of vessel wall, some thrombi in the lumina, and perivascular neutrophilic infiltrate in the mid and deep dermis with leukocytoclasis. Direct immunofluorescence showed granular C3 deposits in the vessel walls and fibrinogen in the lumina.

After this acute episode, complement levels, blood cells and platelet counts returned to normal and the cutaneous lesions faded within 2 weeks.

Since then, new relapses of urticaria-like lesions are still occurring without any obvious trigger factor.

DISCUSSION

The absence of ANA and DNA antibodies as well as the course of the disease rule out the diagnosis of systemic lupus erythematosus which may have been suggested by the urticaria-like lesions (Provost *et al.*, 1980). The presence of urticaria, arthralgia and abdominal pain, the histological finding of leukocytoclastic vasculitis, the low level of first components of complement and the presence of immune complexes are consistent with the diagnosis of hypocomplementaemic vasculitis (Curd *et al.*, 1979; Laurell, Martenson & Sjoholm, 1977; McDuffie *et al.*, 1973; Sissons, Peters & Williams, 1974; Agnello, 1978; Zeiss *et al.*, 1980).

As in the syndrome that McDuffie initially described (McDuffie *et al.*, 1973), there was no evidence of renal damage and the symptoms ended within a few days, but the association with shock, thombocytopenia and leukopenia raises several points.

This man has had recurrent episodes of urticarial vasculitis with collapse, and finally an acute shock syndrome. A recurrent relationship is suggested between the cutaneous and the systemic phenomena. The shock is unlikely to be due to infection because of the absence of any septic focus and the leukopenia, negative blood cultures and rapid response to corticosteroids. A reaginic hypersensitivity type I is unlikely, due to the normal level of IgE. Also the shock did not seem to be due to erythromycin, since this drug had been stopped four days prior to the incident, and an *in vitro* test with this antibiotic was negative.

Therefore, we think that the shock and urticaria could be reasonably explained as manifestations of hypocomplementaemic vasculitis. Other anaphylactic-like symptoms such as laryngeal oedema have been previously described with hypocomplementaemic vasculitis (Zeiss *et al.*, 1980).

The leukopenia and thrombocytopenia may have been a part of the disease too, since they returned to normal levels after the acute episode. The plasma clotting studies rule out an intravascular coagulopathy, and nothing in the past history explains both acute thrombocytopenia and leukopenia.

We believe that the combination of shock, urticaria, leukopenia and thrombocytopenia suggests a unique mechanism involving complement components $C3_a$ and $C5_a$. We have no laboratory data to confirm this idea, but this case report and the *in vitro* tests support this hypothesis. *In vitro* experiments show that anaphylatoxins (C3a, C5a) are able to release histamine. C5a can also induce aggregation and adhesion of polymorph neutrophils (Craddock *et al.*, 1977) and their migration through vascular endothelium. Thus, an excessive release of C5a by complement activation (Marder *et al.*, 1978) might induce a leukopenia, as occurs in patients undergoing haemodialysis. In such patients, complement is activated by the membranes and releases C5a which is responsible for leukopenia (Chenoweth *et al.*, 1980, Jacob *et al.*, 1980). In the dengue shock syndrome, there is more substantial evidence that leukopenia, thrombocytopenia and shock can result from massive complement activation and this is usually attributed to the anaphylatoxins which are generated (Russell & Brandt, 1973). This idea is supported by the reduced concentration of C3 and C5 found in severe cases of dengue haemorrhagic shock (Bokisch *et al.*, 1973).

Perivascular leukocytes in urticarial vasculitis might release their platelet activating factor (Penttinen *et al.*, 1971), resulting in thrombocytopenia and maybe the release of platelet vasoactive amines; these phenomena could be increased by the immune complexes since they modify platelet adhesion (Benveniste, 1974). Thus, the leukopenia and thrombocytopenia could both be due to complex interactions involving anaphylatoxins, leading to this very unusual association of shock with hypocomplementaemic vasculitis.

This rare patient supports but does not prove our hypothesis that anaphylatoxins released in hypocomplementaemic vasculitis may cause 'shock'.

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Juvenile hyaline fibromatosis

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SUMMARY

A 4-year-old boy with juvenile hyaline fibromatosis (systemic hyalinosis) is described. The clinical features, pathology and prognosis of this condition are discussed.

Juvenile hyaline fibromatosis (JHF) or systemic hyalinosis is a rare disease characterized by skin lesions, gum hypertrophy, muscle weakness and flexion contractures of the large joints. Defects in the long bones and punched-out osteolytic lesions have also been described. The skin lesions may consist of multiple large tumours, particularly on the scalp and around the neck, and small pink or pearly papules on the nape of the neck, around the alae nasi and on the trunk.

Sporadic cases have been reported under different titles and the disease was established as the entity juvenile hyaline fibromatosis by Kitano *et al.* (1972). More recently, Iwata *et al.* (1980) have suggested that JHF is a connective tissue disease characterized by increased synthesis of glycosaminoglycans by fibroblasts.

CASE REPORT

The child was born in 1978 in the United Kingdom to unrelated Indian parents. He was born at term by normal delivery and weighed 2·3 kg. There were no significant perinatal problems. His mother had noticed small spots at the sides of his nose at birth and by the age of 18 months the gums had started to enlarge. He was referred to the Paediatric Department at the University Hospital of Wales because of delayed motor development. He did not sit until he was 10 months old and his early locomotion was by means of rolling. When placed in the standing position he was able to walk at 18 months, but he could not get up unaided until the age of 2·5 years. At this age he was unable to climb on to a chair and could only climb stairs on 'all fours'. His brother, born in 1975, is unaffected.

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Examination

At the time of presentation he had small plaques around the alae nasi (Fig. 1) and multiple small pearly papules on the upper lip, the bridge of the nose, the sides and nape of the neck, and in the retroauricular folds and mastoid region where they coalesced (Fig. 2). There was gross gingival enlargement affecting the upper and lower dentition with folds of firm gingiva extending to cover the incisors (Fig. 3).

Power was good in all muscle groups in the legs but he walked with hips and knees slightly flexed and with the feet everted, suggesting functional weakness of the antagonist muscle groups. There was a mild deformity of the knees. There was marked weakness and reduced muscle mass in the shoulder girdle and triceps muscle and he was unable to raise his arms above the head. The remainder of the neurological examination was unremarkable.

Hearing and eyesight were normal and there was no impairment of mental development.

Investigations

Electromyogram, nerve conduction studies and serum creatine phosphokinase levels were all normal. Muscle biopsies from the vastus lateralis showed a normal spectrum of fibre size and the deltoid muscle biopsy showed only a mild increase in variability of fibre diameter with several mildly atrophic fibres and some moderately hypertrophied fibres. The distribution of fibre types in both muscles was normal and the amount of endomysial and perimysial fibrous connective tissue was within normal limits. Intramuscular fibromata were not seen.

A skeletal survey showed an osteolytic lesion in the distal left humerus (Fig. 4) and symmetrical medial defects were seen in the proximal ends of the tibiae and humeri (Figs 5a,b). There were no abnormalities of the nasal bones, skull or spine.

Full blood count and plasma glucose were normal. Testing for glycosaminoglycans on a 24 h specimen of urine was both quantitatively and qualitatively normal.

Histopathology

A skin biopsy from the papular eruption behind the left ear showed deposition of a hyalinised eosinophilic material in the upper dermis filling the papillae (Fig. 6). This material contained only a few cells. The overlying epidermis was thinned. There was a patchy perivascular mononuclear infiltrate in the upper and mid dermis.

Biopsy of the gingiva (Fig. 7) showed extensive deposition of eosinophilic hyaline material which had a greater cellularity and vascularity than the abnormal skin. Embedded within this material were scattered cells which had a 'chondroid' appearance with round or oval margins and clear cytoplasm. The hyaline material was PAS positive and diastase resistant. The matrix did not react to stains for elastic tissue.

Progress and management

During the last 18 months, the disease has progressed. The nose has become deformed due to a bulbous swelling (Fig. 8). Fleshy growths have developed inside both nostrils blocking the airway. These have been removed surgically but recurrence has been noted within 6 months of their excision. Firm, painless swellings have appeared in relation to the distal right radius, around the medial and lateral malleoli of both ankles and on the left supra-orbital ridge. These swellings are seen on the X-rays involving soft tissue but there is no involvement of the underlying bones. Soft swellings with a gelatinous consistency have appeared behind the external ears bilaterally and on the pulp of the right great toe. The gum hypertrophy has interfered with eating. A gingivectomy produced a good result for only a short time, and the

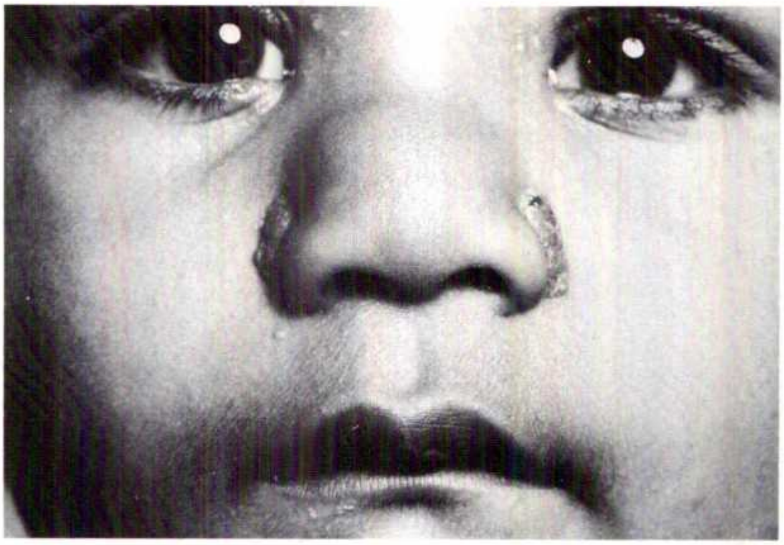

FIGURE 1. Aged 2 years. Small plaques around the alae nasi and papules on the upper lip and bridge of the nose.

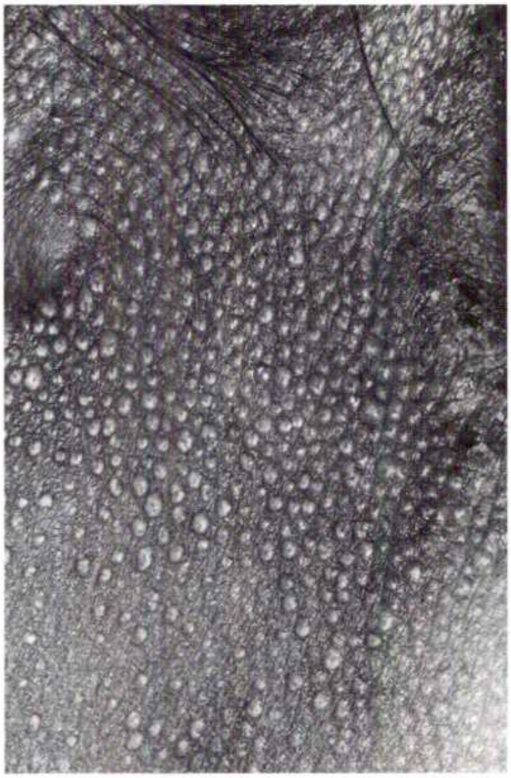

FIGURE 2. Multiple papules coalescing over the mastoid region.

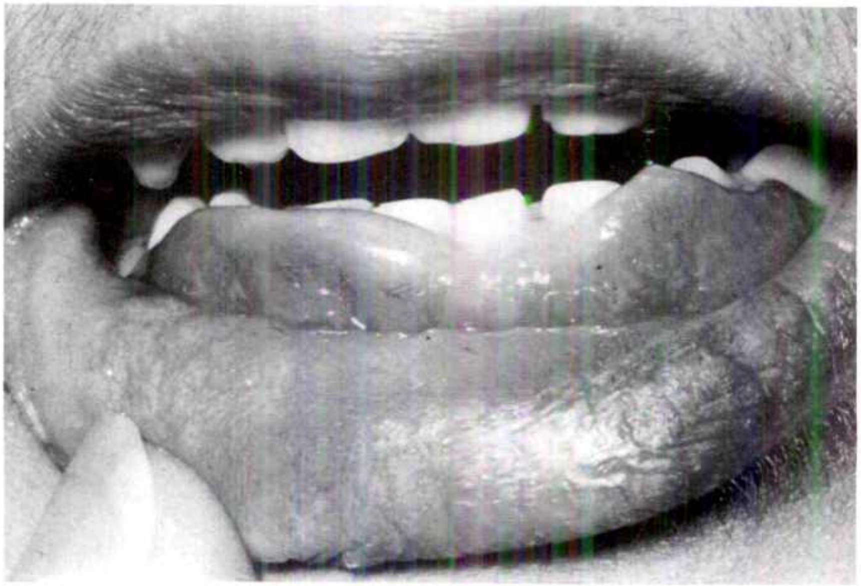

FIGURE 3 Gross gingival enlargement.

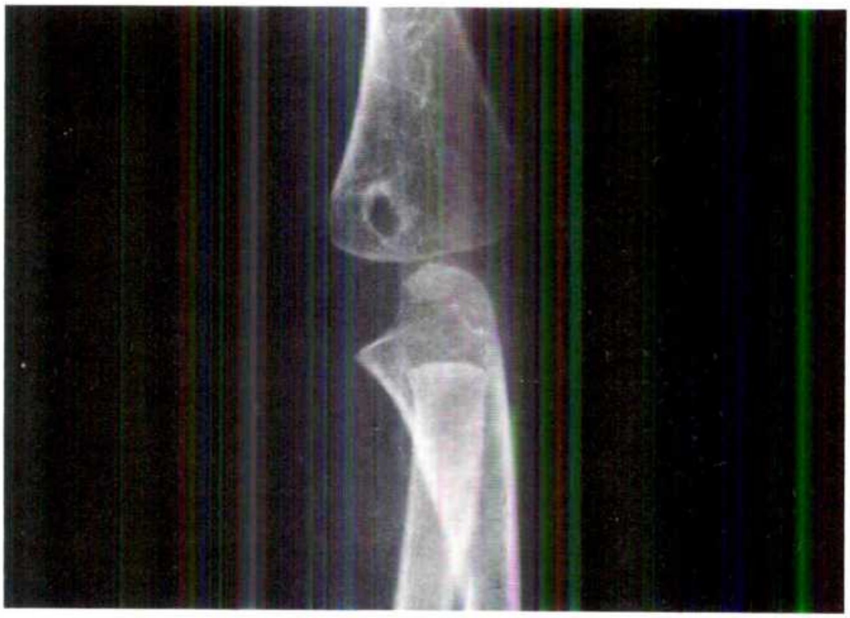

FIGURE 4. Osteolytic lesion, distal left humerus.

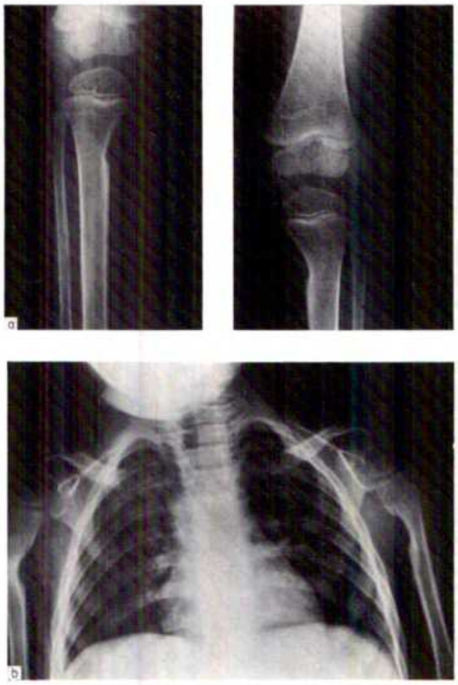

FIGURE 5. Symmetrical cortical defects. (a) proximal tibiae, (b) proximal humeri.

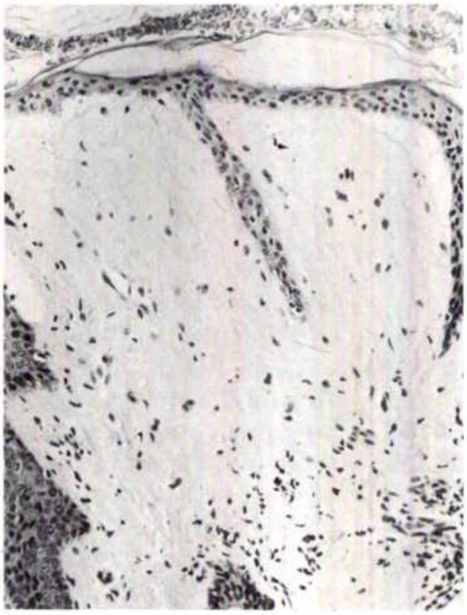

FIGURE 6. Skin biopsy behind the ear showing dermal papillae containing hyalinised amorphous material (H & E, × 212).

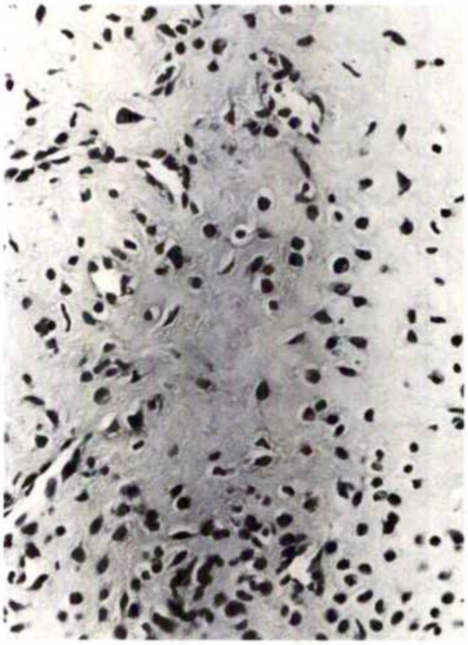

FIGURE 7. Biopsy from gingiva showing many 'chondroid' cells embedded in the hyaline material (H & E, × 424).

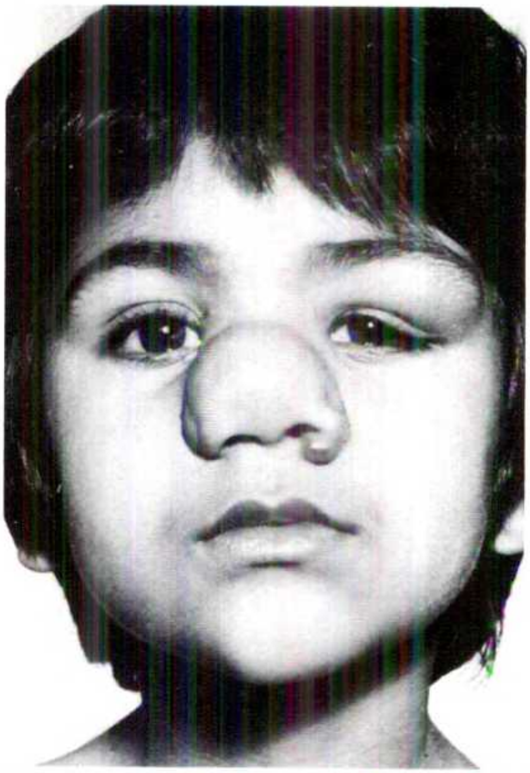

FIGURE 8. Aged 3·5 years. Bulbous deformity of the nose and prominent left supra-orbital ridge. Soft tissue tumour arising from the rim of the left nostril.

operation had to be repeated 18 months later. The papules on the neck and behind the ears have become larger and the tumours at the sides of the nose are now more prominent.

There has been some improvement in the strength of the arms and the shoulder girdle over the past 18 months. His mental development has been normal. The child has received no medication with oral corticosteroids, cytotoxic or antimetabolite drugs.

DISCUSSION

Juvenile hyaline fibromatosis is a disorder characterized by skin lesions, gingival enlargement, joint contractures and bone lesions. It is very rare and occurs sporadically, but the disease has been described in siblings (Kitano *et al.*, 1972; Drescher *et al.*, 1967).

The skin lesions may be of several types. The most common are small, fleshy, pearly papules which tend to occur either singly or in groups on the face and neck, particularly behind the ears and alae nasi as demonstrated in this case and others (Puretić *et al.*, 1962; Ishikawa & Hori 1964; Kitano *et al.*, 1972). Translucent nodules on the pulps of the fingers, behind the external ears and on the tip and columella of the nose have also been described (Atherton, Lake & Wells, 1981). Large subcutaneous tumours may also occur and affect most commonly the scalp, but they have also been described on the trunk and limbs. These tumours may be hard or soft, may be mobile or bound to the underlying fascia and they may ulcerate (Kitano, 1976). Gingival enlargement is a very common finding and may be so severe that the soft tissue extends onto the occluding surface of the teeth.

Joint contractures are often noted and flexion contractures of the fingers, elbows, hips and knees have been described (Puretić *et al.*, 1962; Ishikawa & Hori 1964; Kitano *et al.*, 1972; Kitano, 1976; Atherton *et al.*, 1981).

Although muscle symptoms have been described in this syndrome, the muscle disorder has not been well characterized. The children reported by Ishikawa & Hori (1964), Kitano *et al.* (1972) and Kitano (1976) were small and had poorly developed musculature. The boy reported by Ishikawa & Hori (1964) had a palpable nodule in the tibialis anterior muscle at its proximal attachment. Histologically, the muscle fibres were of an irregular size with loss of striations. Hyaline material separated the muscle fibres.

Delayed motor development was a significant feature in our case. Muscle enzyme studies and electromyography were normal and the muscle biopsy showed mild non-specific abnormalities only. No intramuscular fibromata were detected and there was no deposition of hyaline material in either of the two muscles biopsied. In view of this, and the natural history of improvement of the muscles over the last 18 months, which contrasts with the progress of the disease elsewhere, it would appear likely that the child has a generalized mild congenital myopathy distinct from a myopathy related to the hyaline fibromatosis.

Bone lesions, demonstrated radiologically, have been reported. Puretić *et al.* (1962) described osteolysis of the distal phalanges of the fingers and toes which progressed to almost complete destruction of these phalanges. Kitano (1976) reported punched-out osteolytic lesions in the skull, the left femur and the left tibia and destruction of the proximal ends of both humeri with absence of the humeral heads. Atherton *et al.* (1981) reported osteolytic lesions in the distal phalanges of the great toes and the left lateral malleolus. Ishikawa & Hori (1964) described a medial cortical defect in the proximal left tibia similar to that seen in our patient. Tissue taken from this area in their patient showed fragmentation of collagen fibres separated by hyaline material. This hyaline infiltrate of the collagen was similar to that seen in the muscle and skin sections (Ishikawa & Hori, 1964). Guilhou & Meynadier (1982) described also symmetrical medial defects at the proximal ends of the femora, humeri and tibiae in a 12-year-old boy.

The treatment of this condition is unsatisfactory. Excision of tumours may be followed by recurrence. Joint contractures have been helped by treatment with cortisone, ACTH and physiotherapy, but the tumours do not respond to radiotherapy (Puretić *et al.*, 1962).

Knowledge of the natural history of this condition is provided by only a few case reports and hence there is little available information about its prognosis. We have contacted several authors personally to learn of the progress of their reported patients.

The patient reported by Puretić *et al.* (1962) is now 33 years old, and is severely physically handicapped but mentally normal. There are severe flexion contractures of the fingers, elbows and knees with ankylosis of these joints. Many skin nodules persist and continue to ulcerate. Some nodules have regressed spontaneously. The gingival enlargement has not improved.

The boy reported by Ishikawa & Hori (1964) is now 21 years old and is very small (height 80 cm; weight 21 kg). Joint contractures have resulted in severe immobility of the limbs. Skin tumours have not regressed spontaneously and one tumour recurred after resection.

The surviving child reported by Kitano *et al.* (1972) is 22 years old and the boy reported by Kitano (1976) is now 16 years old. Both patients are mentally normal. They continue to develop occasional large tumours which ulcerate frequently. Excision has proved a valuable treatment allowing ulcerated areas to heal. Kitano found that intralesional steroid injections sometimes reduced the size of lesions in their early stages when they were soft. Joint contractures have been very disabling and the beneficial effect of capsulotomy (Kitano, 1976) was only temporary. Osteolytic lesions have extended particularly in the phalanges.

From this follow-up data it would appear that any significant spontaneous regression of this disease is unlikely. There have been no reports of the effect of long-term treatment with corticosteroids, cytotoxic or antimetabolite drugs. Principal pathological features of these tumours are the presence of 'chondroid' cells embedded within an amorphous eosinophilic ground substance. The larger tumours contain much more ground substance and the smaller newly-developed tumours are more cellular (Kitano, 1972). Ishikawa *et al.* (1979) examined the hyaline material ultrastructurally and showed that the hyaline material was composed of ruthenium red-positive granules, filaments and cross-banded structures, indicating the presence of a glycosaminoglycan or glycoprotein. Using a ruthenium red staining method and selected enzymatic digestion with chondroitinase, hyaluronidase and pronase, they demonstrated that the cross-banded structures and granules consisted of a chondroitin sulphate-rich proteoglycan and/or glycoprotein. Collagen fibrils were decreased in number and thinner than mature fibrils, and the skin collagen content of the tumour was decreased. Elastic fibres were not present in the hyalinised areas.

Kitano *et al.* (1972) demonstrated numerous intracytoplasmic granules within the 'chondroid' cells. These granules were similar to those seen in the ground substance and were present in the rough endoplasmic reticula and in Golgi vesicles. These findings suggest that the hyaline material is produced by these cells and released to the extracellular space. Iwata *et al.* (1980) have demonstrated increased chondroitin synthesis by skin fibroblasts cultured from the tumour tissue. Therefore, these results suggest that juvenile hyaline fibromatosis is a connective tissue disorder characterized by an aberrant synthesis of glycosaminoglycans by fibroblasts.

ACKNOWLEDGMENTS

We are indebted to Professor Ishikawa, Gunma University, Japan, Professor Kitano, Osaka, Japan and Dr. Stefanija Puretić, Zagreb, Yugoslavia for sending detailed information on the progress of their patients. We are grateful to Dr R.J.M. Lane, Department of Neurology, University of Newcastle-upon-Tyne, who reported the muscle biopsy and to Dr Murray Walker, Department of Oral Medicine, Welsh National School of Medicine, who provided the sections from the gingival biopsy, and to Dr I. Grossman for his help with translation.

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Balloon cell melanoma: a case report

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SUMMARY

A rare malignant tumour, the balloon cell melanoma, is reported and the histogenesis is discussed. The unusual cytological changes described may pose difficulty in establishing the malignant status of balloon cell melanoma.

CASE REPORT

A 42-year-old woman presented with a pigmented lesion on the right forearm which had been present since childhood. Two months prior to presentation this had become very irritant, and occasionally bled on scratching.

Histological examination of haematoxylin and eosin-stained sections revealed a tumour occupying the papillary and upper reticular dermis, with considerable attenuation and erosion of the overlying epidermis (Fig. 1). The tumour cells, which were closely packed, possessed a voluminous spumous cytoplasm and varied considerably in size and nuclear characteristics (Fig. 2). In general the cell nuclei were centrally situated but occasionally they were adjacent to the cell membrane; many nuclei were large and hyperchromatic and a number of mitoses were evident. Many of the tumour cells were seen infiltrating the epidermis and were also present in the serous crust overlying the eroded epidermis. At the lateral margins of the tumour mass, small nests of more typical melanocytes, albeit with some cytological aberration, were present at the dermo-epidermal junction. A dense mononuclear cell infiltrate comprising predominantly lymphocytes and a lesser number of histiocytes was observed at the lower border of the tumour mass. Fontana silver staining for melanin revealed its presence within macrophages and lying free within the dermal infiltrate.

DISCUSSION

The presence of large foamy vacuolated cells in benign naevi was reviewed by Gartman (1960), who found an incidence of 1·78%. These cells predominate in the balloon cell naevus and its malignant counterpart, the balloon cell melanoma.

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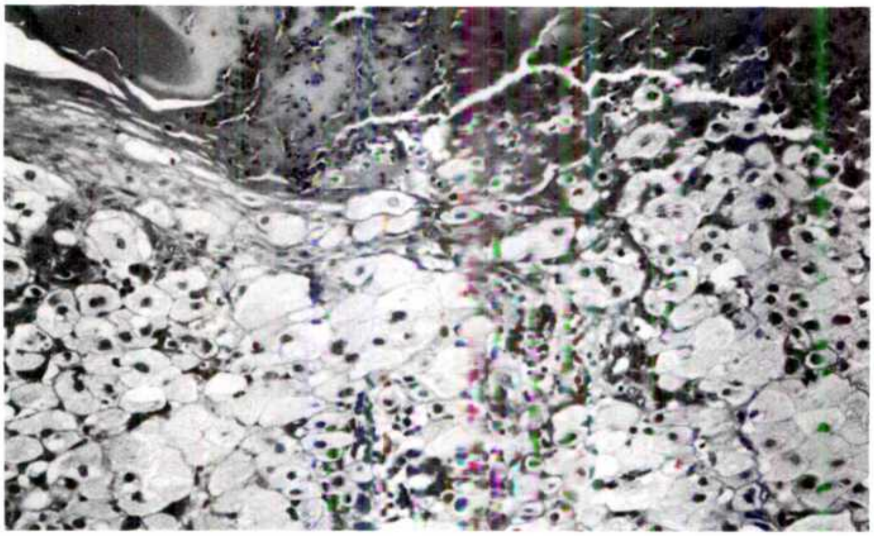

FIGURE 1. Invasion and erosion of the overlying epidermis by balloon cells.

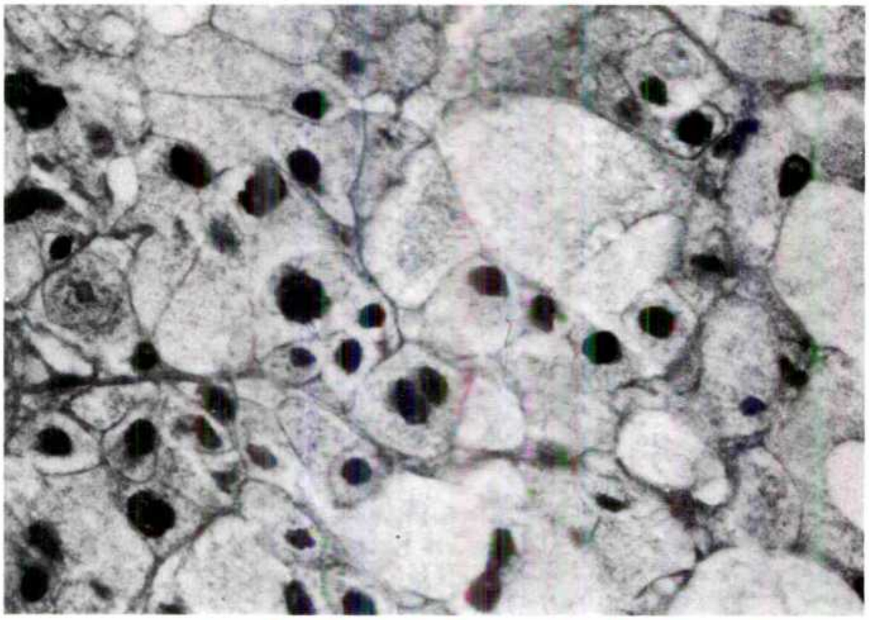

FIGURE 2. Balloon cells showing variation in size and nuclear characteristics.

The paucity of reports in the literature suggests that balloon cell melanoma of the integument is very rare (Gardner & Vazquez, 1970; Ranchod, 1972; Hula, 1973) although the occurrence of the lesion as an ocular tumour has been more frequently reported (Riley, 1974; Rodriguez & Shields, 1976; Jakobiec *et al.*, 1979). In the cases recorded, including our own, the lesions have been clinically indistinguishable from melanoma with conventional histological patterns.

The nature and origin of balloon cells has been the subject of much debate (Schrader & Helwig 1967). Ultrastructural studies of balloon cell melanoma indicate that balloon cells are indeed of melanocytic origin. A defect in melanosome formation, with the failure of premelanosomes to develop an internal structure, allows subsequent accumulation of large membrane-bound vesicles. These vesicles displace cellular organelles and account for the reticulate appearance of the cytoplasm (Ranchod, 1972).

The balloon cell naevus has a characteristic topographical appearance easily recognizable at low magnification. Schrader & Helwig (1967) emphasized the diagnostic difficulties which may be encountered with malignant lesions of this type. The spectacular cytology may 'swamp' the tell-tale features of malignancy such as epidermal invasion. In particular the balloon cells and any conventional melanocytic cells should be carefully examined for features of atypia including cellular pleomorphism, nuclear hyperchromatism and high mitotic index, if malignant lesions are not to be overlooked.

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. *Comment*

DIAGNOSING EPIDERMOLYSIS BULLOSA

The diagnosis of epidermolysis bullosa (EB) usually depends on careful clinical, genetic and histological assessment. This approach will help to get the right answer in about three out of four cases. In the remaining quarter, diagnostic difficulties will occur because there will be confusing physical signs, unhelpful histology and no family history. Electron microscopy has added considerably to our understanding of the pathogenesis of different forms of EB and is especially useful in diagnosis by demonstrating the precise level of cleavage in the skin, and other important ultrastructural features. It helps to distinguish different types of disease which may show considerable clinical and histological overlap. Biochemical studies, particularly of skin collagenase, have also contributed to our insight of underlying disease mechanisms and have proved useful in differentiating two main kinds of dystrophic EB.

Before trying to diagnose the more difficult cases, one should have some knowledge of an acceptable classification of EB, the best way to take a biopsy, and which laboratory procedures to use for examining the skin.

Classification

The classification of this complex and heterogeneous group of diseases now depends to a large extent on first determining the level of blistering (see Table I). Thus, in all forms of EB simplex, the split occurs in the epidermis. In the junctional forms, the skin separates in the lamina lucida of the dermo-epidermal junction (for a review of the ultrastructure of the dermo-epidermal junction, see Briggaman & Wheeler, 1975a); whereas in the different types of dystrophic EB, the blister occurs in the superficial dermis, below the basement membrane. In answer to the question 'How many variants are there?' most dermatologists will probably recognize five. These would comprise two simplex forms, namely the localized (Weber–Cockayne) and the widespread (Koebner) variants; one junctional type (Herlitz or letalis) and two dystrophic variants—one autosomal recessive and the other dominant.

In his painstaking treatise on EB, Gedde-Dahl (1971) pointed out that there were several forms of the disease that previously had not been properly differentiated. Now, with further clinical and genetic studies in his own patients together with collaborative electron microscopy performed by Anton-Lamprecht and her co-workers in Heidelberg, Gedde-Dahl (1981) would like us to believe that there are no less than sixteen types of EB. Is this the true picture and should we therefore accept the sixteen variants? It is still too early to tell. Independent observations are needed to confirm or refute the Gedde-Dahl and Anton-Lamprecht classification. It would seem justified to include the herpetiform variant (Dowling & Meara, 1954) of EB simplex since this appears to be a separate entity, characterized by rather typical clinical features, and, in the opinion of Anton-Lamprecht & Schnyder (1982) by a specific ultrastructural abnormality in the epidermis. It will be disputed, however, whether Bart's syndrome (congenital localized absence of skin) (Bart *et al.*, 1966) and Mendes da Costa disease should be listed as specific forms of EB simplex. Mendes da Costa disease is not regarded as a mechano-bullous disorder. Wojnarowska, Eady & Wells (1983) feel that localized absence of skin can be a presenting feature in different forms of EB.

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TABLE I. Classification of genetic forms of epidermolysis bullosa

Type	Inheritance	Level of separation	Other features
Simplex (Epidermolytic)			
Generalized (Koebner)	AD*	Basal cells	
Hand and foot (Weber–Cockayne)	AD	Basal and suprabasal cell	
Herpetiform (Dowling–Meara)	?	Basal cells	Abnormality of tonofilaments
Ogna (Gedde-Dahl)	AD	Intraepidermal	Bruising; linkage with glutamic pyruvic transaminase locus
Mottled pigmentation (Fischer & Gedde-Dahl)	?AD	Intraepidermal	
Junctional			
Lethal (Herlitz)	AR†	Lamina lucida	Abnormality of hemidesmosomes
Non-lethal generalized (Hashimoto *et al.*)	AR	Lamina lucida	
Non-lethal inverse (Anton-Lamprecht & Gedde-Dahl)	AR	Lamina lucida	
Dystrophic (Dermolytic)			
Generalized (Hallopeau–Siemens)	AR	Below basal lamina	Anchoring fibril disorder‡; Increased skin collagenase and collagenolysis
Localized	AR	Below basal lamina	
Inverse (Gedde-Dahl)	AR	Below basal lamina	
Pasini	AD	Below basal lamina	Albopapuloid lesions; abnormality of GAG metabolism
Cockayne–Touraine	AD	Below basal lamina	

* Autosomal dominant; † autosomal recessive; ‡ present in all forms of dystrophic disease.

The main problem with junctional EB is to know what the prognosis is in different cases. It used to be assumed that all patients with the lethal (Herlitz) variant would die soon after birth, but there have been cases reported by Pearson, Potter & Strauss (1974) surviving several years, albeit with severe disease. The case of Ridley & Levy (1968) had widespread non-scarring EB and died at the age of 35 years. Electron microscopy was not used to confirm the diagnosis which was thought to be the Herlitz variant. This notion is supported by a probable autosomal recessive inheritance and because a brother died of epidermolysis bullosa in early infancy. In general, however, the lethal nature of Herlitz disease tends to be constant within affected families.

There are now further documented cases of non-lethal junctional EB in adults (Hashimoto, Schnyder & Anton-Lamprecht, 1976; Hintner & Wolff, 1982) which suggest both phenotypic and genotypic differences compared with the Herlitz variant. Moreover, the 'inverse' types of junctional and dystrophic EB, inherited as recessive traits and characterized by lesions predominantly on the trunk rather than acrally, should also be delineated from other forms of the disease.

Clinical features

These are described in the major text books and are important in differentiating the various forms of EB. As a rule of thumb, the simplex types do not scar or involve the nails or mucous membranes. The lesions in the junctional variants also do not leave scars but can result in permanent atrophic changes, and frequently involve the mucosae and cause shedding of nails, whereas the dystrophic forms are associated with scarring, milia and nail dystrophy. In severe recessive dystrophic EB (Hallopeau–Siemens) there is widespread blistering, the oral mucous membrane and teeth are affected and there may also be oesophageal lesions with scarring and strictures. Nail dystrophy and loss are characteristic. Dominant dystrophic EB is usually less severe than the recessive variants. The blisters tend to be localized to areas of maximum trauma (for example, the knees, ankles and lower legs) and the mucous membranes and teeth are usually spared. In some cases it is not possible to make a clinical distinction between the recessive and dominant dystrophic forms. The former may be relatively mild and the latter more severe than usual. The Pasini form of dominant dystrophic EB is characterized by the presence of albopapuloid lesions, usually on the trunk. These lesions are not specific to the Pasini variant for we have seen them in a patient with junctional disease.

The greatest difficulty is encountered in the neonate with widespread blisters and no family history of EB. It may be impossible to distinguish the junctional from the dystrophic forms on clinical grounds. Severe involvement of the buttocks, with erosions on the back of the scalp and of the peri-ungual areas, is suggestive of Herlitz disease. Furthermore, the widespread simplex disease may be so severe in early life that it too, cannot be confidently diagnosed without electron microscopy. Cases of EB simplex have been reported with severe oral involvement and even milia and atrophic changes resembling superficial scars (Baker, 1982; Yacoub *et al.*, 1979).

Taking a biopsy

Since diagnosis depends on establishing the level of cleavage in the skin, care must be taken to select an appropriate site for biopsy and to use a technique causing the least artefact. The best site is the edge of a fresh blister. The histological picture will be difficult to interpret and even misleading if the blister is more than 12 hours old, when healing had begun. If no fresh lesions are available for biopsy, blistering may be induced in a predilection site by gentle friction with a pencil rubber. In certain variants, such as herpetiform simplex and dominant dystrophic disease, blisters may be very difficult to provoke experimentally. In such cases, therefore, it will be necessary to search for a new, spontaneously arising lesion. In the lethal and recessive dystrophic types of EB, blisters are liable to arise anywhere and biopsy trauma commonly causes separation of epidermis from dermis.

The preferred type of biopsy is either a shave or an ellipse. Since the pathological changes are either in the epidermis or just underneath, shallow biopsies will suffice. The use of a punch is not recommended because the applied torque will frequently cause total separation and even loss of the epidermis. Cooling the skin with ice, prior to biopsy, does not appear to prevent biopsy-associated separation in severe dystrophic disease.

Light microscopy

For most forms of EB, 'epidermolysis' is a misnomer, for lysis of the epidermis occurs only in EB simplex.

Conventional histology will not distinguish between the junctional and dystrophic forms. Even if special stains (e.g. periodic acid-Schiff) of the basement membrane zone are used, the diagnosis is often unclear. In EB simplex the cleavage may be so low in the epidermis (less than a micrometre above the basal lamina) that the true changes will be missed on examination of 5 or 6 μm thick paraffin-embedded sections. With 1-μm thick plastic (epoxy resin) sections it is sometimes possible to resolve the fragments of basal keratinocytes resting on the blister base. If the changes of EB simplex are more obvious, then basal and suprabasal cytolysis can be recognized by conventional light microscopy.

Electron microscopy

Transmission electron microscopy is the most accurate and safest method for establishing the diagnosis in all forms of EB and should be used routinely. With standard techniques, the diagnosis can be made 5 days after taking the biopsy. With more rapid processing it should be possible to get results within 2 days. The diagnosis depends on determining the level of cleavage in relation to the dermo-epidermal junction (Pearson, 1962) (see Table I). These findings are consistent for any type of the disease.

In EB letalis, in addition to the split in the lamina lucida, there is an abnormality of hemidesmosomes (Arwill, Bergenholtz & Thilander, 1968; Hashimoto *et al.*, 1976a). These structures are relatively sparse and may lack a sub-basal dense plate. A similar abnormality may occur in the non-lethal (benign) form of junctional EB (Hashimoto, Schnyder & Anton-Lamprecht, 1976) although detailed morphometric studies in one patient showed structurally and numerically normal hemidesmosomes in the presence of widespread and marked reduction in dermo-epidermal adhesion (Tidman & Eady, 1982).

Abnormalities of anchoring fibrils have been noted in both dominant dystrophic (Anton-Lamprecht & Schnyder, 1973) and recessive dystrophic EB (Briggaman & Wheeler, 1975b). Apparent differences in anchoring fibrils between the two main dominant dystrophic variants have also been reported (Hashimoto, Anton-Lamprecht & Gedde-Dahl, 1975; Hashimoto *et al.*, 1976a). However, recent quantitative studies suggest that enumeration of anchoring fibrils may not be capable of distinguishing between recessive and dominant dystrophic EB (Tidman & Eady, 1983). Tidman *et al.* (1982) have also shown that anchoring fibrils are irregularly distributed in normal skin thus underlining the need for adequate controls in studies of this kind.

Immunofluorescence (IMF) microscopy

By studying the localization in fresh blisters of antibodies directed against normal components of the dermo-epidermal junction, Hintner *et al.* (1981) and Kero *et al.* (1982) showed that it was possible to discriminate between the main variants of EB. Baker (1982) used IMF localization of bullous pemphigoid antisera in the diagnosis of EB simplex, and Schachner, Lazarus & Dombitzer (1977) showed that the staining characteristics with bullous pemphigoid antiserum were different in junctional compared with dystrophic EB. It is argued that IMF microscopy, which can be performed on frozen sections, is quicker and simpler than electron microscopy (Hintner *et al.*, 1981). However, rapid electron microscopy may turn out to be less tedious than IMF which necessitates using multiple conjugates. Furthermore IMF, unlike electron microscopy, will not allow any distinctions to be made between different types of junctional or simplex EB.

Biochemistry

Bauer, Gedde-Dahl & Eisen (1977) demonstrated increased amounts of collagenase in non-blistered skin in recessive dystrophic and junctional EB but not in the simplex or dominant dystrophic types. Later, Bauer & Eisen (1978) found increased synthesis of collagenase *in vitro* by recessive dystrophic EB fibroblasts but not by cells from other variants of the disease. The albopapuloid (Pasini) variant of dominant dystrophic EB has been differentiated from other forms of dominant or recessive dystrophic EB because fibroblasts from affected patients display an abnormality of glycosaminoglycan metabolism—probably increased synthesis (Bauer, Fiehler & Esterly, 1979). While providing insight into the pathogenesis of EB, biochemical analysis has not generally been employed as a first-line technique in diagnosis. However, given the difficulty in classifying certain patients with dystrophic EB, assessment of their skin fibroblast collagenase or glycosaminoglycan synthesis may help to place them into the recessive or dominant group.

Prenatal diagnosis

This was first achieved in EB letalis (Rodeck, Eady & Gosden, 1980) and later in the Hallopeau–Siemens type of dystrophic EB (Anton-Lamprecht *et al.*, 1981). Small skin samples are taken at 18 to 20 weeks' gestation during fetoscopy and examined by electron microscopy. At this stage of fetal development the ultrastructure of the dermo-epidermal junction is similar to that of the neonate and the level of cleavage can be established. Blisters are not evident *in utero* but the biopsy trauma is sufficient to cause cleavage either within the lamina lucida in EB letalis, or below the basement membrane in dystrophic EB. Rodeck *et al.* (1980) noted that the hemidesmosomes were abnormal in a fetus with EB letalis. Prenatal diagnosis has now been attempted in several cases at risk from EB (Eady *et al.*, 1982). Raised levels of maternal serum alphafetoprotein in the mid-trimester have been described in association with two cases of EB simplex (Yacoub *et al.*, 1979; Cowton *et al.*, 1982).

Epidermolysis bullosa acquisita (EBA)

EBA is now widely accepted as a distinct blistering disorder with an apparent immunological basis (Roenigk & Pearson, 1981). Cleavage occurs below the basement membrane and immunoelectron-microscopical studies (Nieboer *et al.*, 1980; Yaoita *et al.*, 1981) have shown deposition of immuno-reactants within the superficial dermis. EBA is unrelated to the genetic variants of EB but may have to be considered in the differential diagnosis of acquired diseases such as pemphigoid, amyloidosis and porphyria cutanea tarda.

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Ophthalmological side-effects of 13-cis-retinoid therapy

SIR, Recent findings by Milson *et al.* (*British Journal of Dermatology* 1982, **107,** 491) suggest that 13-cis-retinoid does not cause marked changes in tear secretion (Schirmer test) and tearfilm break-up time (BUT), although the authors recognise that these tests are crude and difficult to reproduce. We have evidence however suggesting that 13-cis-retinoid does cause a decrease in tearfilm BUT.

Seven patients were examined ophthalmologically about 3 months after starting 13-cis-retinoid (3 patients 1 mg/kg/day; 4 patients 0·5 mg/kg/day) for acne conglobata and again about 3 months after stopping the drug. No abnormalities were found. The basal tear secretion was assessed by the Schirmer test without use of an anaesthetic and appeared unchanged during and after therapy. Two patients had a very low level of basal tear secretion (1 & 2, see Table 1). Patient 1 complained of dry eyes and slight visual blurring during therapy. Patient 2 remained symptom free. Patient 7, the only one to wear contact lenses, was unable to continue to do so until about 3 months after discontinuation of the drug.

The BUT was also measured three times in one session on each eye (Table 1). The difference between BUT during and after therapy with 13-cis-retinoid was highly significant ($P < 0{\cdot}01$, using a t test for paired data on the mean for both eyes). These data could very well explain why patients cannot continue to wear contact lenses during 13-cis-retinoid therapy.

TABLE 1. Tearfilm break-up time (BUT) in seconds on the right and left eye during and after 13-cis-retinoid therapy

	BUT during therapy			BUT after therapy		
Patient	Right	Left	Mean	Right	Left	Mean
1	8	8	8	16·3	5·3	10·8
2	5	8·6	6·8	7·3	14·3	10·8
3	7·3	12	9·6	31	31	31
4	15	12	13·5	19	18·3	18·6
5	4·3	6·3	5·3	20	31	25·5
6	14·6	16	15·3	≥40	≥40	≥40
7	14	14	14	≥40	≥40	≥40

The tearfilm on the hydrophobic cornea comprises three layers. The inner-most layer apposed to the cornea consists of mucus; the middle layer consists of tear-fluid (water and salt); the outer layer consists of lipids. In view of the fact that 13-cis-retinoid causes a definite fall in sebum excretion rate, a possible explanation of decrease in BUT during 13-cis-retinoid therapy could be a reduction of lipid content of the tearfilm, thereby increasing evaporation. However, a reduction of mucus secretion of the goblet cells of the conjunctiva would also reduce BUT. A change of humidity could also affect BUT. The first series of measurements was performed in April, May and June, the second series in October and November. Because of central heating the humidity might have decreased in the examination room in winter, during the second series of measurements. Consequently, such a change of environmental humidity is unlikely to affect our conclusion that 13-cis-retinoid probably does cause a decrease in tearfilm BUT.

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Trauma-induced bullae: the presenting feature of systemic amyloidosis associated with plasma cell dyscrasia

SIR, We were interested in the case reports by Holden *et al.* (*British Journal of Dermatology* 1982, **107**, 701) of two patients with a plasma cell dyscrasia who developed a bullous eruption with amyloid deposits along the epidermal basement membrane. They also found basement membrane deposition of immunoglobulin similar to that observed in pemphigoid.

We have recently seen a Caucasian male aged 64 years who initially presented to the nephrologists in August 1979 with a nephrotic syndrome and was diagnosed with no major criteria of myeloma as having a plasma cell dyscrasia and renal amyloidosis. He was successfully treated with four short courses of melphalan and prednisolone, the last of which finished in January 1982. Six months later he developed blistering of his skin in response to even minor trauma and was referred to the Dermatology Department. On examination, the bullous eruption was extensive and involved mainly frictional areas on his limbs and around his waist. Some bullae were clear and others were blood-filled, and they arose from normal-looking skin. Many blisters had ruptured giving rise to large areas of denuded skin. Nikolsky's sign was positive. Purpura were easily induced by shearing injury. There were no oral lesions and no other abnormalities were found on general examination. Suction-induced blistering time on the inner arms was markedly reduced to 11 min from the normal of 60–90 min, under controlled conditions. Histological examination of involved skin revealed a subepidermal bulla with little reactive inflammation. Congo-red P and Sirius red stains showed amyloid in the blood vessels deep in the dermis but not, as in the case of Holden *et al.*, in the basement membrane. In addition no amyloid could be demonstrated in clinically uninvolved skin. Immunofluorescence studies failed to detect immunoreactants either in the basement membrane zone or in the vessel walls, both in involved and uninvolved skin. Electron microscopy revealed a split involving the basal lamina.

We report this case, both because of certain similarities to the recently reported cases and also because it differs in several ways:

(i) Amyloid was demonstrated only in the blood vessels in the deep dermis and not at the basement membrane.

(ii) Direct immunofluorescence was negative.

(iii) There was a long interval (3 years) between the diagnosis of systemic amyloidosis and the development of his bullous disease.

This patient is being treated with prednisolone with remission of his skin lesions and a detailed case report will follow. We suggest that the bullous eruption is not directly related to the presence of cutaneous amyloid but involves a change, as yet unidentified, in the collagen.

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Pharmacological variability of crude coal tar

SIR, Lowe *et al.* (*British Journal of Dermatology* 1982, **107**, 475) showed a decrease of DNA synthesis 4 h after topical application of coal tar. They cited previous publications which supported their work. They ignored the majority of reports which disagreed with their findings and which reported an increase in mitotic rate labelling index or epidermal thickness (Jambon, 1909; Combes, 1943; Kaidbey & Kligman, 1974; Elgjo & Larsen, 1973; Saldfeld & Orth, 1965; Wrench & Britten, 1975; Foreman *et al.*, 1979; Sarkany & Gaylarde, 1976).

Furthermore, their observations on the pharmacological variability of coal tar are not new. This was first demonstrated by Downing & Bauer (1948) and confirmed in later studies (Wrench & Britten, 1975; Sarkany & Gaylarde, 1976). In addition we reported data on the effect of various coal tar fractions on epidermal thickness and showed that coal tar and certain of its fractions could suppress epidermal thickening induced by a topically applied irritant.

Coal tar stimulates epidermal turnover in normal skin. However, coal tar causes suppression of epidermal hyperplasia in psoriasis (Fry & McMinn, 1968) and of thickening induced by irritants. It is therefore reasonable to postulate that components with both irritant and anti-phlogistic activity are present. Part of this suppressive activity was present in an acid-soluble fraction that has minimal irritant properties (Sarkany & Gaylarde, 1976).

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Reply

SIR, I am most grateful for the above letter from Drs. Sarkany and Gaylarde. I was aware of reports showing proliferative responses to topical coal tars in normal epidermis and did not choose to intentionally ignore these reports. In fact, our paper quoted one study by Lavker *et al.* (1981) who reported an early hyperplastic effect of tar on normal skin followed by epidermal thinning after 40 days application.

We do not know the mechanisms of action of coal tar used in the therapy of different skin diseases. There are numerous agents that are effective in psoriasis and as an early effect suppress epidermal DNA synthesis rates. Anthralin, for example, will suppress epidermal DNA synthesis as an early event after treatment of normal epidermis (Lowe & Breeding, 1981), but also as a later event will produce a hyperproliferative response. This is also true of UV-B irradiation.

Our paper on the pharmacological variability of coal tar therefore reported the use of an alternative bioassay to investigate one biological effect of crude coal tar and a purified tar product. It should also be noted that the crude coal tar tested in our studies was obtained from the same source using apparently the same distillation temperatures and procedures. It was clear from our results that the distillate fractions from this source had significantly different activities in the bioassay of epidermal DNA synthesis suppression in the hairless mouse. This suggests the possibility of variable therapeutic activity, even when crude coal tar is obtained from one supplier. Because of space restraints, an exhaustive list of references was not possible and I am grateful for the additional references on coal tars published with their letter.

I would like to thank Drs. Sarkany and Gaylarde for their observations which bring more fully into perspective the extremely complex effects of coal tars on the skin.

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Book Reviews

Tissue Growth Factors. Edited by R. Baserga (1981) Berlin: Springer-Verlag. (No. 57 in Handbook of Experimental Pharmacology Series). Pp 630. Price $154.

Books are a little like people. Some are slim, pretty and superficial, others are weighty, dependable and authoritative. This book falls into the latter class. Biology owes much to Professor Renato Baserga who over the years has produced a series of publications of immense value to research workers in the field of cell kinetics and cell culture. The present volume contains seventeen excellent reviews with the overall theme of tissue-derived growth control substances. There is an introductory chapter by Baserga himself which starts the ball rolling with a discussion on some of the events that take place before and during cell division. Your reviewer found this of particular interest as it contains interesting data on the changes in cell size before and during mitosis. An important contribution follows from a very eminent cell culturist, Professor Ham, who has written encyclopaedically on the effects of changes in the media on cell growth *in vitro*. Subsequent chapters deal with individual tissue-derived growth control substances. Of particular interest to the practising skin biologist are chapters on epidermal growth factors and chalones. They are both written by acknowledged experts (Professors G. Carpenter and O.H. Iversen) and stand as definitive monographs on these subjects. Nonetheless, I would have preferred a modicum of doubt in the section on epidermal chalone in which it is stated 'It is now established beyond reasonable doubt that aqueous extracts of epidermis contain substances with specific growth inhibitory effect on keratinocytes.' Clearly the chapter on chalones has to be read with the author's phrase 'The author . . . works within the paradigm that chalones exist,' very much in mind. Other sections of more general interest include those on proteases, androgenic factors, somatomedins, and glucocorticoids—all of more than passing interest to those interested in skin. This is a work of immense scholarship. It demands a place in all medical school libraries and laboratories concerned with cell culture and cell kinetics but only the wealthier scientists will want to purchase a copy personally.

R.Marks

Clinico-Pharmacological Models for the Assay of Topical Corticoids. H. Wendt and P.J. Frosch. (1982) Basel: Karger. Pp. 61. Price D.M.40.

Even those of us who speak out against topical corticosteroids in public, use them (sparingly, I hope!) in the privacy of our clinics. They have been and will continue to be, an important part of the practice of dermatology as well as a focus for controversy. Anything which extends our knowledge and understanding of these drugs should be welcomed. For this reason I was delighted to see this slim and well-presented booklet. Curiously enough I cannot remember a previous volume similarly devoted to the methods and models used for the assay of topical corticosteroids. It is essentially a guide to 'would-be' clinical investigators of one sort or another and in this sense is quite useful. Clearly the authors have extensive experience of the experiments they describe and the book is liberally laced with their own results. In fact the publication is really a collection of 'minipapers' documenting their studies with groups of corticosteroids.

Useful though it is, the book has faults. The title itself is somewhat misleading. Topical corticosteroids are its focus, and not topical 'corticoids'. Furthermore the title suggests that it reviews all the methods available rather than documenting the authors' own experiments. The book is copiously illustrated but many of the pictures, although of good quality, are of little value. Do we really need pictures of a blister and a pale, vasoconstricted area covering whole pages? Similarly there seems little point in having quite so many pictures of dressings and tubes of creams. A final minor grumble stems from the English style. I realize that English is not the first language of the authors but surely the publishers could have made the flow of words a little less stodgy?

These points aside, I enjoyed reading it. I believe that it will be useful also for many clinical dermatologists to learn some of the practicalities of the tests on the preparations they prescribe.

R.Marks

British Journal of Dermatology (1983) **108**, 632.

News and Notices

CIRD Symposium: Advances in Skin Pharmacology, Sophia Antipolis, October 1983

The 4th CIRD Symposium will be held at Sophia Antipolis, near Nice, on 21–23 October 1983. The aim of the Symposium will be to examine methods linking cell and animal experiments with clinical experience. The presentations will be divided into three sessions: New *in vitro* approaches; Recently developed animal models; Clinical aspects. The Symposium will be organized by M.Greaves, L.Juhlin, M.Prunieras, H.Schaefer and B. Shroot. Abstracts of up to 300 words should be sent as soon as possible and not later than May 15, 1983 to: Prof. Hans Schaefer, Centre International de Recherches Dermatologiques, F-06565 Valbonne, France. Further information is available on request.

Practical Skin Pathology Course, New York, August 1983

A course on Practical Skin Pathology approved by the A.M.A. and the A.A.D. will be given at Grossinger's, Liberty, New York, 28 August to 2 September 1983. This is a comprehensive and practical review of Dermatopathology, sponsored by the Department of Dermatology, New York Medical College-Metropolitan Hospital Center, New York, U.S.A. There will be 38 formal lectures, 8 supervised microscope sessions of 400 unknown slides, and 20 additional hours for optional independent study of the slides. For further information, please write to Martin H.Brownstein, M.D., Skin Pathology Course, 2 Jordan Drive, Great Neck, New York 11021 or phone (516) 829-8578.

British Society for Dermatopathology

A one-day educational meeting will be held prior to the main B.A.D. Meeting on Monday 27 June 1983, at Kensington Town Hall, London. Invited speakers will include prominent European dermatopathologists. For further information please contact Dr R.Russell Jones, Secretary, British Society for Dermatopathology, St John's Hospital for Diseases of the Skin, Lisle Street, Leicester Square, London WC2H 7BJ.

The Wycombe Prize 1983

Nominations are invited for this prize which is offered for an outstanding contribution to dermatology published from a district general hospital or non-teaching centre in the United Kingdom. For regulations please contact the Hon. Secretary, B.A.D. Office, 7 John Street, London WC1N 2ES.

The Whimster Prize

Nominations are invited for the above prize set up to commemorate the late Dr Ian Whimster. It will be awarded on the basis of work (including theses) published in the preceding 3 years in the field of experimental pathology of the skin. Nominations to be sent to the Hon. Secretary, B.A.D. Office, 7 John Street, London WC1N 2ES.

Consultant Appointments

The following appointments have recently been made.

Dr W. Branford, Consultant Dermatologist to the Kettering District.
Dr A.Chu, Senior Lecturer in Dermatology, The Hammersmith Hospital, London.
Dr T.P.Cutler, Consultant Dermatologist to the East Suffolk District Health Authority, based at Ipswich.
Dr A.S.Highet, Consultant Dermatologist to York and Scarborough Health Districts.
Dr J.M.Mahood, Consultant Dermatologist to the Northampton & Kettering Health District.
Dr R.J.Mann, Consultant Dermatologist to the Swindon District Health Authority.
Dr I.R.White, Consultant Dermatologist at St John's Hospital for Diseases of the Skin, London.

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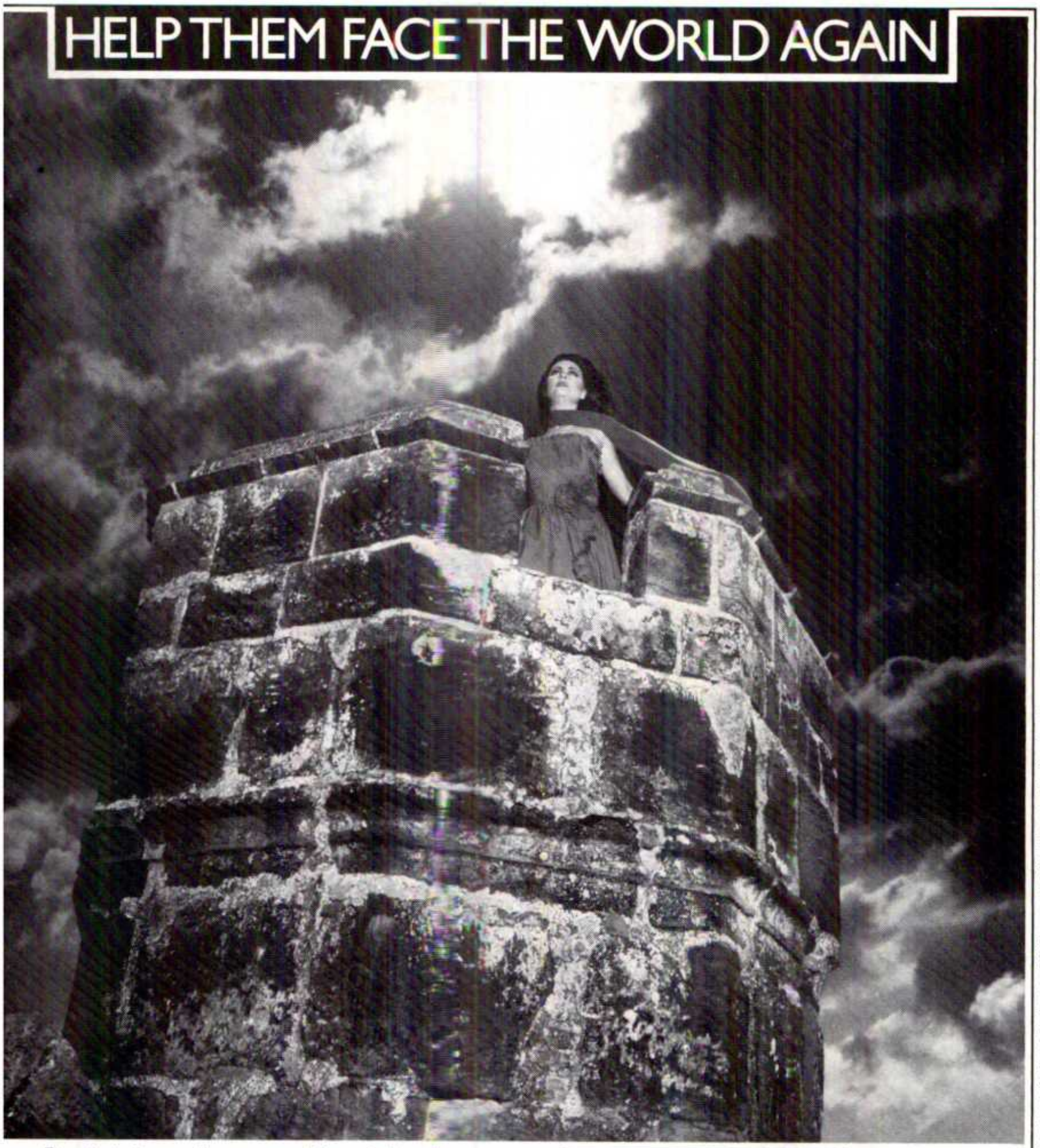

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Reference: 1. Medicine 1977; 32: 1857.

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Diseases of the Nails and their Clinical Management

Edited by **R. Baran** MD, *Head of Department of Dermatology, General Hospital, Cannes,* and **R.P.R. Dawber,** MA, MB, ChB, FRCP, *Consultant Dermatologist, Oxford Hospitals and Clinical Lecturer in Dermatology, University of Oxford*

This important title will be an essential reference for all clinicians involved in the diagnosis and management of diseases of the nails. It presents a comprehensive account of the structure and function of the nails, and of the clinical and therapeutic aspects of all nail disease, including surgical treatment where appropriate. Chapters have been contributed by a team of authors of international renown and the book is lavishly illustrated with colour plates to assist in diagnosis.

Contents: Embryology, anatomy and physiology *R.P.R. Dawber;* Physical signs *R. Baran;* Investigations *R.P.R. Dawber;* The nail in childhood and old age *R. Baran;* Fungal diseases *R.J. Hay;* The nail in dermatological disease *R. Baran;* The nail in systemic disease *D. Fenton and J.D. Wilkinson;* Occupational abnormalities and contact dermatitis *R.J.G. Rycroft;* Genetic abnormalities *L. Juhlin;* Surgical treatments (and skin tumours) *H. Bureau, E. Haneke and R. Baran;* Cosmetics *E. Brauer.*

Summer 1983. 500 pages, 300 illustrations (200 in colour). About £48.00

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Essentials of Skin Disease Management

D.J. Barker MA, MB, MRCP, *Lecturer in Dermatology, University of Leeds and Honorary Registar in Dermatology, The General Infirmary at Leeds*

and **L.G. Millard** MB, MRCP, *Consultant Dermatologist, Rotherham Area Health Authority and Clinical Lecturer, University of Sheffield*

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1979. 96 pages, 25 illustrations. Paper, £5.50

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NOTICE TO CONTRIBUTORS

The *British Journal of Dermatology* publishes original articles on all aspects of the normal biology, and of the pathology of the skin. Originally the Journal, founded in 1888, was devoted almost exclusively to the interests of the dermatologist in clinical practice. However, the rapid development, during the past twenty years, of research on the physiology and experimental pathology of the skin has been reflected in the contents of the Journal, which now provides a vehicle for the publication of both experimental and clinical research and serves equally the laboratory worker and the clinician. To bridge the gap between laboratory and clinic the Journal publishes signed editorials reviewing, primarily for the benefit of the clinician, advances in laboratory research, and also reviews of recent advance in such aspects of dermatology as contact dermatitis, therapeutics and drug reactions. Other regular features include book reviews, correspondence and society proceedings. The Journal is the official organ of the British Association of Dermatologists and also of the Netherlands Association for Dermatology and Venereology, but it attracts contributions from all countries in which sound research is carried out, and its circulation is equally international.

Papers accepted become the copyright of the Journal. This Journal is covered by *Current Contents, ISI/BIOMED, Science Citation Index* and *ASCA*.

Manuscripts should be sent to the Editor, Dr J.L.Burton, Dermatology Department, Bristol Royal Infirmary, Bristol BS2 8HW. They should be typewritten on one side of the paper only, with a wide margin, be double spaced, and bear the title of the paper, name and address of each author, together with the name of the hospital laboratory or institution where the work has been carried out. The name and full postal address of the author who will be responsible for reading the proofs should be given on the first page. A duplicate copy, complete with figures, tables and references, should be sent, since in many cases two referees are consulted. The author should keep a carbon copy of his manuscript. The Editorial Board reserves the right to make literary corrections.

Abbreviations and units. Full names with uncommon abbreviations must be given with the first mention: new abbreviations should be coined only for unwieldy names and should not be used at all unless the names occur frequently. In the title unusual abbreviations should be identified, in the introduction and discussion they should be used sparingly. Abbreviations of units should conform with those shown below.

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kilogram(s)	kg	centimetre(s)	cm	millilitre(s)	ml
milligram(s) (10^{-3}g)	mg	second(s)	s	gravitational acceleration	***g***
microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm^3	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
hours(s)	h	milliequivalent	mmol		

These and other abbreviations and symbols should follow the recommendations of: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Editors and Authors* (1977) The Royal Society of Medicine, London.

Illustrations should be referred to in the text as 'Figs', and be given Arabic numbers, and should be marked on the back with the name(s) of the author(s) and the title of the paper. Where there is any possible doubt as to the orientation of an illustration, the top should be marked with an arrow. Each figure should bear a reference number corresponding to a similar number in the text. Photographs and photomicrographs should be unmounted glossy prints and should not be retouched. Colour illustrations will be accepted when found necessary by the Editor, although the author will be expected to bear the cost. Diagrams should be on separate sheets; they should be drawn with black ink on white paper on feint-ruled graph paper and should be about twice the size of the final reproduction. Lines should be of sufficient thickness to stand reduction. Each illustration should be accompanied by a legend clearly describing it; these legends should be grouped on a separate sheet of paper.

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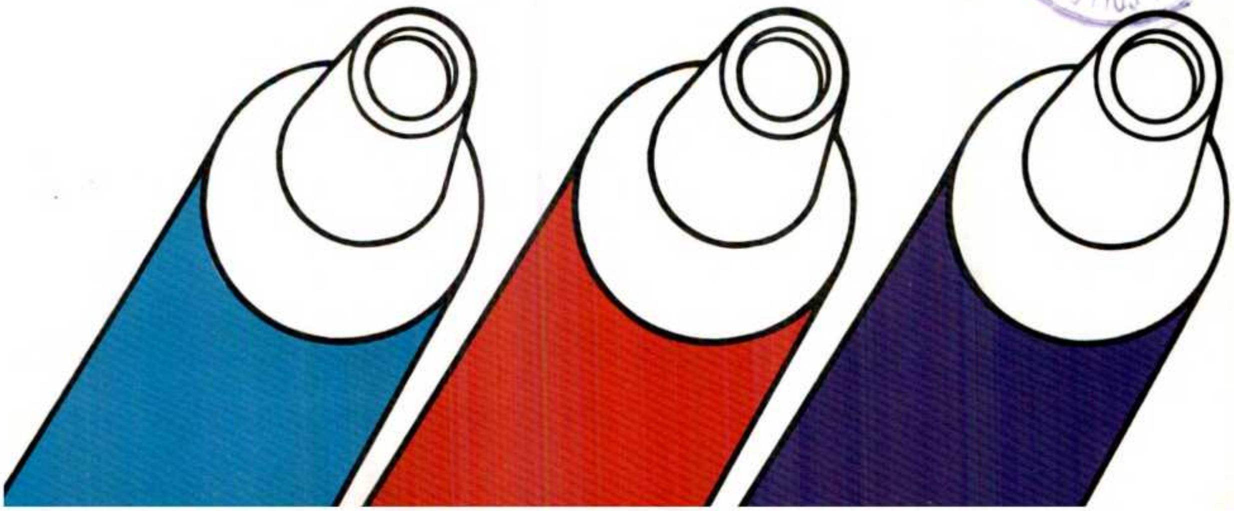

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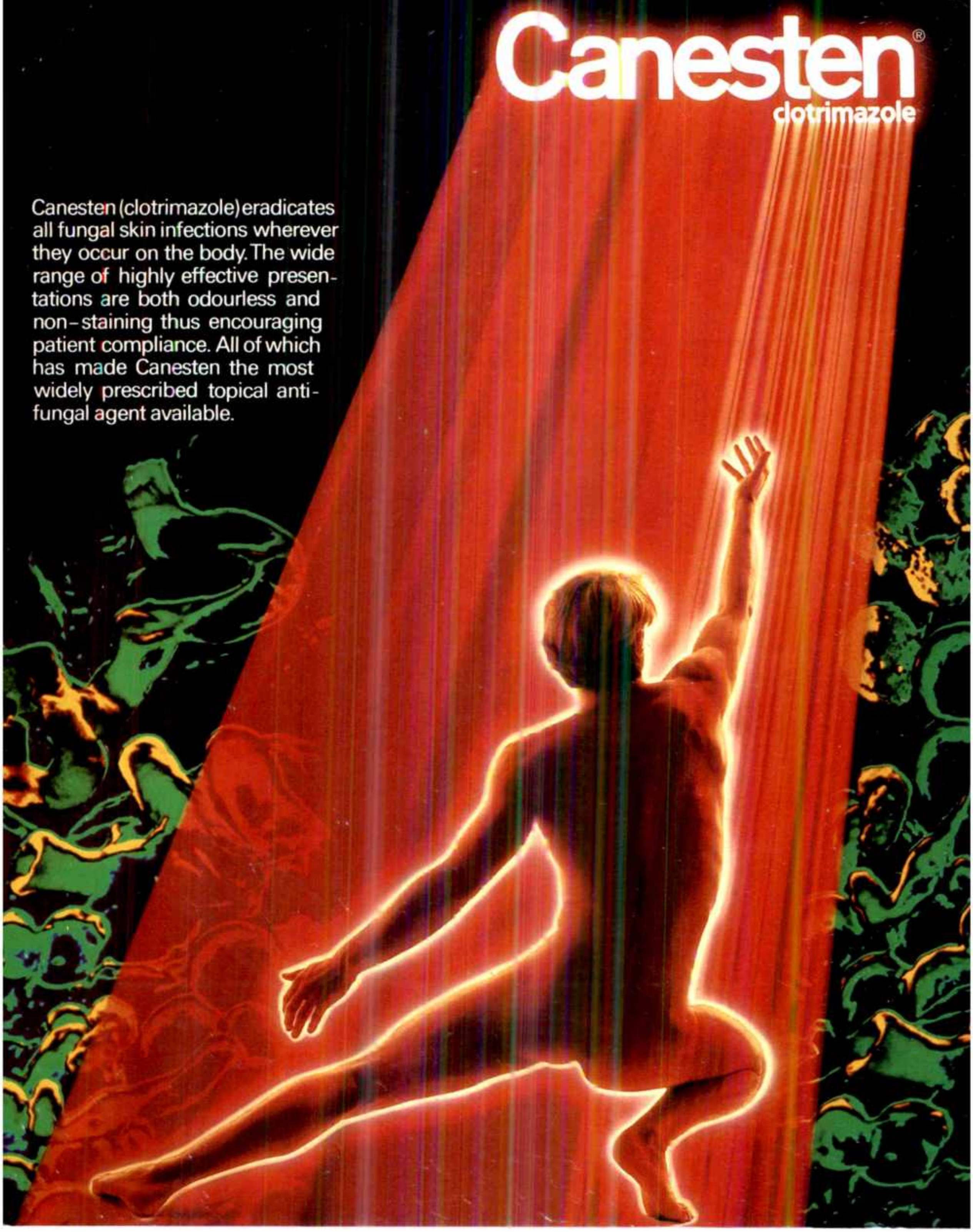

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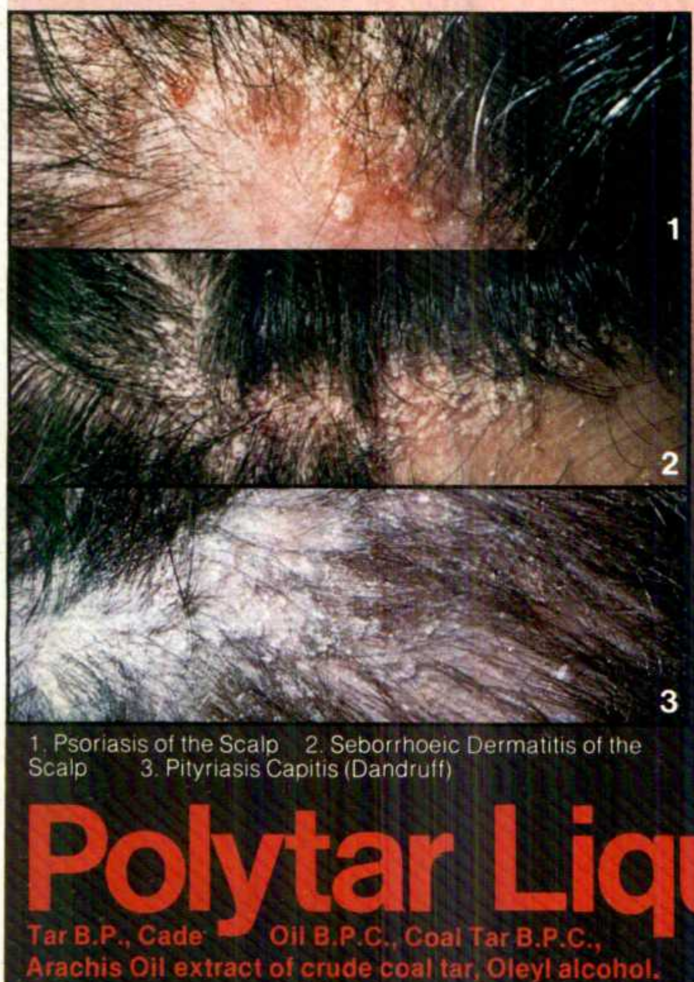

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Dosage and Administration The hair should be wetted and sufficient applied to produce an abundant lather. The scalp and adjacent areas should be vigorously massaged with the fingertips. The hair should then be thoroughly rinsed and the procedure repeated.
Polytar Liquid or Polytar Plus should be used once or twice weekly.

Contra-indications, warnings, etc. Nil

Pharmaceutical Precautions Store in a cool place

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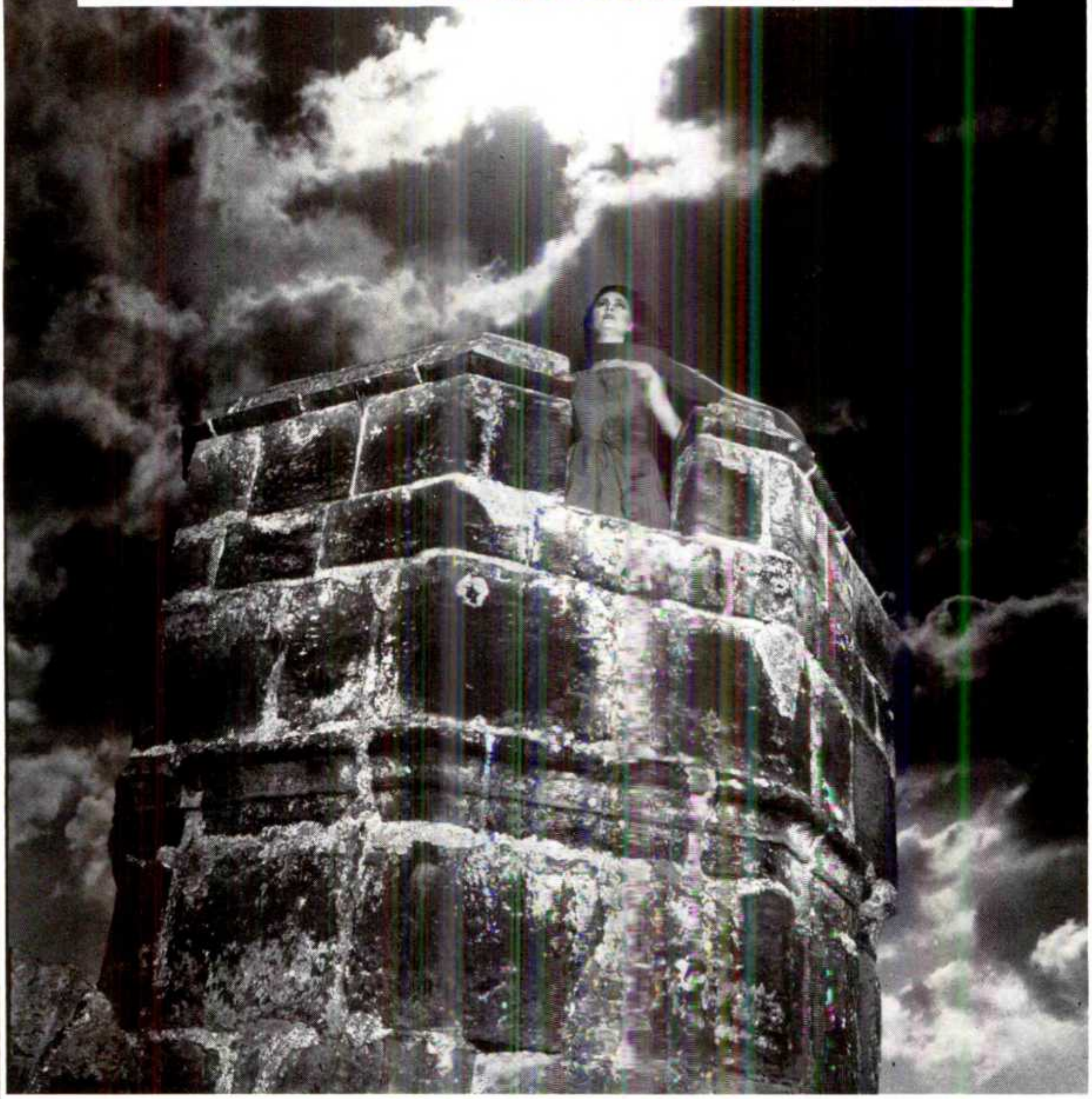

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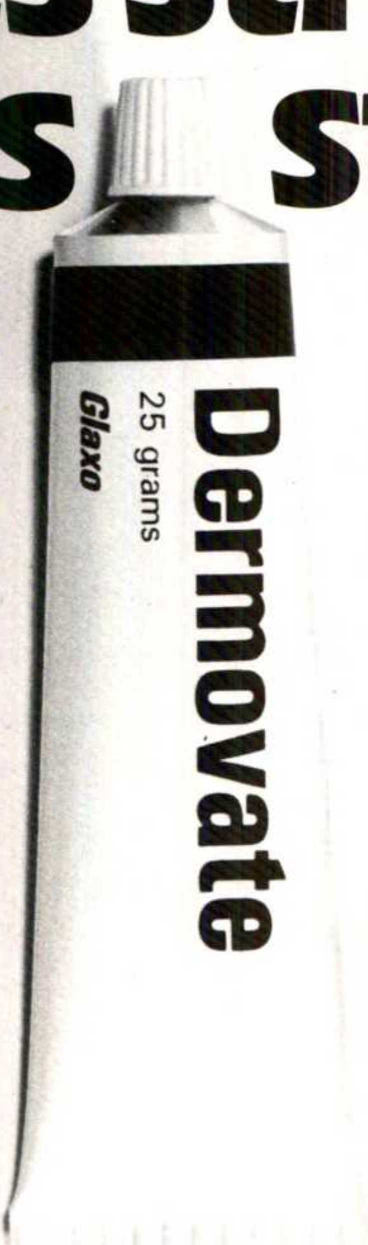

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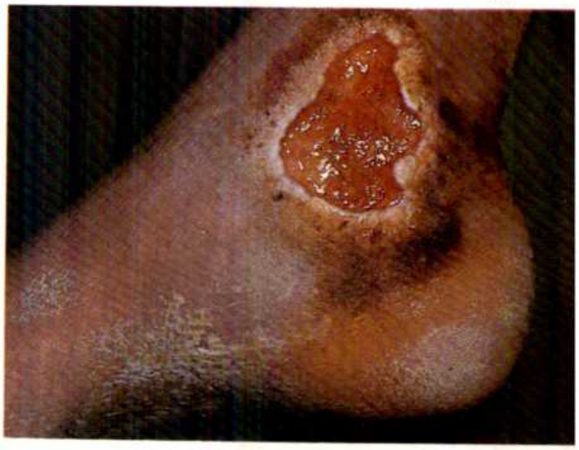

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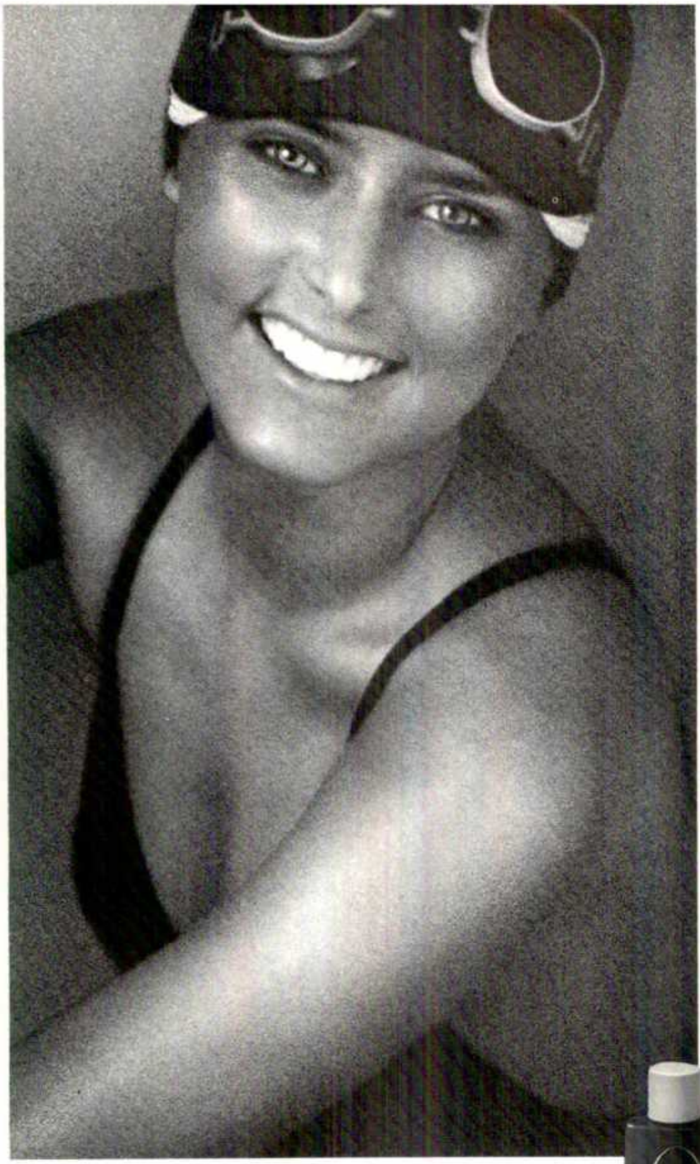

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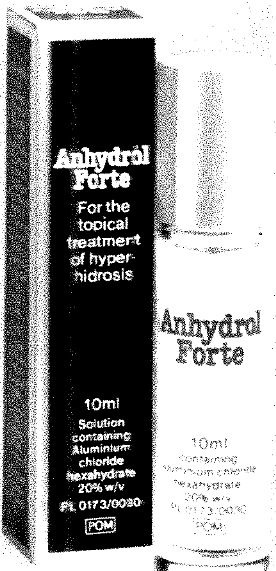
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British Journal of Dermatology (1983) **108**, 633–638.

Clinical and Laboratory Investigations

Early diagnosis of infantile seborrhoeic dermatitis and atopic dermatitis—clinical features

VICTORIA M.YATES, REBECCA E.I.KERR AND RONA M.MACKIE*

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Accepted for publication 12 October 1982

SUMMARY

In a prospective study of thirty-seven infants presenting with dermatitis in early infancy it was found that pruritus, a positive family history of atopy, and the age of onset of skin lesions were of little value in distinguishing atopic dermatitis from infantile seborrhoeic dermatitis. The most useful distinguishing features were the increased incidence of lesions on the forearms and shins in atopic dermatitis, and in the axillae in infantile seborrhoeic dermatitis. The onset of skin lesions solely in the napkin area favoured a diagnosis of infantile seborrhoeic dermatitis, but when more than one site was involved the distinction was less clear. Infantile seborrhoeic dermatitis carried a much better prognosis than atopic dermatitis. In thirty-six out of thirty-seven infants the definitive diagnosis could be made with confidence on clinical grounds by the age of 1 year, but was often difficult at first presentation in early infancy.

The distinction between seborrhoeic dermatitis and atopic dermatitis in the very young infant is frequently difficult. Indeed not all clinicians believe they are distinct entities and Vickers (1980) prefers to term infantile seborrhoeic dermatitis the 'seborrhoeic pattern of infantile eczema.'

We agree with Beare & Rook (1979) that the prognosis of infantile seborrhoeic dermatitis and atopic dermatitis is generally so very different that it is well worthwhile to try to differentiate between them at an early age. We have therefore carried out a prospective study with the aim of defining clinical manifestations of value in the early diagnosis of infantile seborrhoeic and atopic dermatitis. In this study we have not attempted to separate 'napkin psoriasis' from seborrhoeic dermatitis although some authors feel there is justification for doing so as there may be a higher incidence of psoriasis in these infants and their relatives (Warin, 1967; Neville & Finn, 1975).

METHODS

Thirty-seven infants under 6 months of age, presenting consecutively with extensive infantile dermatitis were studied.

Correspondence: Dr V.M.Yates, The Skin Hospital, Quay Street, Manchester, M3 3HL.

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Information regarding age at onset of skin lesions, site of onset, spread of eruption and evidence of pruritus was obtained from the parents. The method of infant feeding was noted and if an infant had been breast-fed further enquiries were made regarding duration and possibility of complementary cows' milk feeds at any stage. In infants who had commenced mixed feeding any suspected or observed reactions to foods were recorded. Personal and family history of atopic disease was obtained in this group of patients and in a control group of thirty-seven infants attending a well baby clinic. A positive family history of atopy was recorded when one or more first or second degree relatives had a positive family history of asthma, eczema or allergic rhinitis.

Infants were fully examined and the distribution and morphology of the eruption noted. In view of the obvious difficulties of assessing pruritus in this age group, infants were regarded as having pruritus only if excoriations were obvious or scratching was observed by parents or clinicians. Swabs were taken from the skin of the napkin area, axillae and trunk for isolation of bacteria, *Candida* species and *Herpes simplex*.

At first presentation infants were assigned to one of three categories: (a) definite diagnosis of atopic dermatitis, (b) definite diagnosis of infantile seborrhoeic dermatitis and (c) diagnosis uncertain. A definite diagnosis of atopic dermatitis was made only if infants satisfied Hanifin & Rajka's criteria (1980). This required having at least three of the following basic features: pruritus, facial and extensor involvement, chronic or chronically relapsing dermatitis and personal or family history of atopy. Also, three or more minor features such as xerosis, tendency to cutaneous infections, early age of onset, cheilitis, facial pallor or erythema, food intolerance etc., were required. A firm diagnosis of infantile seborrhoeic dermatitis was made in those infants who had erythematous scaling lesions affecting the scalp, napkin area and flexures, usually in an otherwise well and contented infant.

An initial treatment regimen consisting of exposure of affected body areas, topical non-fluorinated corticosteroids and emollients was instigated, usually on an in-patient basis. Thereafter infants were seen once or twice monthly depending on clinical progress. At the end of the first year of observation the diagnosis was reassessed in the light of clinical progress and clinical findings were correlated with the diagnosis. Twenty-five out of thirty-seven infants have now been followed for a minimum period of 2 years.

Statistical methods used for analysis of clinical data were the χ^2 test with Yates' correction and Fisher's exact probability test where appropriate.

RESULTS

We studied twenty-three males and fourteen females with a mean age at presentation of 14 weeks (range 3–24 weeks). At first presentation five infants satisfied Hanifin & Rajka's (1980) criteria for diagnosis of atopic dermatitis and in twelve a firm diagnosis of infantile seborrhoeic dermatitis was made. Thus in twenty infants the differentiation between atopic and infantile seborrhoeic dermatitis could not be made with certainty when the patients were first seen. When the infants were reviewed at the age of 1 year, a firm clinical diagnosis had been reached in thirty-six out of thirty-seven patients. Nine satisfied the criteria for diagnosis of atopic dermatitis. Twenty-seven had been diagnosed as having infantile seborrhoeic dermatitis. The remaining infant, as yet undiagnosed, has had erythrodermic episodes. Clinical features found at presentation and during follow-up were reviewed and correlated with the final diagnosis as follows.

Age at onset of skin lesions

There was no significant difference between the mean age of onset of skin lesions in each group of infants, this being 4·9 weeks (range 3–23 weeks) in infantile seborrhoeic dermatitis and 5·4 weeks (range 2–12 weeks) in atopic dermatitis.

Site of onset

In infants with atopic dermatitis the face and napkin area were the first sites to be affected in six infants, the face and scalp in two infants and the napkin area alone in the remaining infant. However in the group with infantile seborrhoeic dermatitis, the eruption commenced in the napkin area in twenty-one out of twenty-seven patients. The face and scalp were the first affected sites in four other infants and the scalp and trunk respectively were the sites of onset in the remaining two infants.

Distribution and morphology of lesions

The distribution of skin lesions in atopic dermatitis is shown in Table 1. Seborrhoeic infants had a significantly increased incidence of lesions in the axillae compared to the atopic infants ($P < 0{\cdot}001$) and atopic infants had a similarly increased incidence of lesions on the forearms and shins compared to the seborrhoeic group ($P < 0{\cdot}001$). Frequency of involvement of other sites was similar in the two groups. Morphologically, lesions were often alike, comprising erythema, scaling and microvesicle formation. Two infants with infantile seborrhoeic dermatitis had lesions on the trunk resembling guttate psoriasis.

Pruritus

Obvious pruritus was not apparent before the age of 6 weeks but was found in nearly a third (29%) of infants with seborrhoeic dermatitis, although it was significantly more common in atopic dermatitis, where it was apparent in 89% of the group. Although this difference between the two groups was statistically significant ($P < 0{\cdot}01$), pruritus in infancy is obviously difficult to assess objectively.

TABLE 1. Comparison of the distribution of skin lesions in infantile seborrhoeic dermatitis and atopic dermatitis

	Infantile seborrhoeic dermatitis (n = 27)		Atopic dermatitis (n = 9)		
Site Affected	No. patients affected	%	No. patients affected	%	P
Scalp	26	96	7	77	N.S.
Face	20	74	9	100	N.S.
Axillae	24	88	2	22	< 0·001
Forearms and shins	5	19	9	100	< 0·001
Napkin area	27	100	9	100	N.S.

N.S. = not significant

TABLE 2. Prevalence of atopy in first and second degree relatives of infants with atopic dermatitis or seborrhoeic dermatitis and control infants

Group	No. with family history of atopy	%	P
Atopic dermatitis (n = 9)	5	55	$< 0\cdot05$
Infantile seborrhoeic dermatitis (n = 27)	9	33	N.S.
Control infants (n = 37)	5	14	

N.S. = not significant ($P > 0\cdot05$)

Atopy in relatives

The frequency of occurrence of a positive family history of atopy in infants with seborrhoeic and atopic dermatitis and control infants is shown in Table 2. A third of the seborrhoeic group and just over half of the atopic group had at least one family member with atopy compared with 14% of the control group.

Subsequent course of the disease

Resolution of the skin lesions seen at presentation occurred after 2–4 weeks with appropriate therapy as previously described. The clearance times did not differ significantly between the atopic and seborrhoeic groups, although relapse rates were quite different. All infants in the atopic group had relapsed before the age of 1 year and four patients who subsequently proved to have the most severe and persistent atopic dermatitis developed a recurrence of skin lesions a few weeks after clearance of the eruption seen at presentation. Two other infants had moderately severe atopic dermatitis and in three the clinical picture had been that of localized mild atopic dermatitis.

In contrast the group with infantile seborrhoeic dermatitis had a much better prognosis, twenty-four out of twenty-seven having no further skin lesions apart from mild napkin erythema. Three infants experienced a transient second episode. One child developed infantile acne at the age of 9 months.

Skin cultures

The bacterial flora isolated from infants with atopic dermatitis and those with seborrhoeic dermatitis was similar. *Staphylococcus aureus* was by far the most common isolate, being found in eighteen out of twenty-seven (66%) of the seborrhoeic group and in six out of nine (66%) of the atopic infants. *Candida albicans* was isolated from 19% of patients with infantile seborrhoeic dermatitis compared with 11% of infants with atopic dermatitis. The slightly higher culture rate in the seborrhoeic infants does not necessarily mean that the organism is a pathogen. *Herpes simplex* was cultured from one child with infantile seborrhoeic dermatitis.

Influence of breast feeding

Three infants in the atopic group developed their skin lesions at 3 to 4 weeks of age while being exclusively breast-fed. Five infants with infantile seborrhoeic dermatitis were also breast-fed at the time of onset of their skin lesions. In these infants breast feeding does not therefore appear to have delayed or prevented the onset of their skin disease.

Food intolerance

Only one patient with atopic dermatitis had clinical evidence of definite food intolerance, developing perioral erythema after eating eggs. None of the seborrhoeic group gave a history of food intolerance.

DISCUSSION

Features that are commonly thought to distinguish atopic from infantile seborrhoeic dermatitis are: age of onset of skin lesions, distribution and morphology of eruption, presence or absence of pruritus, family history of atopy and prognosis (Beare & Rook, 1979).

Although it is generally considered that atopic dermatitis rarely begins before 2 months of age (Roth & Kierland, 1964; Champion & Parish, 1979) and that infantile seborrhoeic dermatitis begins almost invariably between the second and tenth weeks (Beare & Rook, 1979), we found the time of onset to be similar in the two groups. This feature is therefore not of value in differential diagnosis in our series.

Thirty-three percent of infants with seborrhoeic dermatitis had a family history of atopic disorders in their immediate relatives. This did not differ significantly from the incidence in our control group and was similar to the figure of 30% quoted for the incidence of atopy in close relatives of normal persons (Champion & Parish, 1979). Therefore, while we found a positive family history of atopy in a significantly higher percentage of our atopic infants (55%), clearly a positive family history of atopy may not always be as valuable in differentiating between infantile seborrhoeic and atopic dermatitis as some authors have suggested (Rajka, 1975; Brauner, 1979).

We do not recognize napkin psoriasis as a separate entity but include this as a form of seborrhoeic dermatitis. When the site of the eruption was solely in the napkin area this favoured a diagnosis of seborrhoeic dermatitis. However, when skin lesions commenced in two or more areas simultaneously, for example scalp and napkin area, the distinction between seborrhoeic and atopic dermatitis was less clear. Scaling of the scalp was frequently seen in the atopic group, while involvement of the cheeks, usually said to be a hallmark of atopic dermatitis in infancy (Rajka, 1975; Champion & Parish, 1979), was as common in infantile seborrhoeic dermatitis. Seborrhoeic infants had a significantly increased incidence of lesions in the axillae, and atopic infants had a similarly increased incidence of lesions on the forearms and shins. This difference in the distribution of the eruptions was the most significant distinguishing feature in our series.

Obvious pruritus was rarely observed before the age of 6–8 weeks and was found in nearly a third of the seborrhoeic infants, although it occurred significantly more commonly in our atopic group, where 89% had pruritus. The absence of pruritus in very young infants is therefore not helpful in differential diagnosis and as the presence of pruritus at a later date is not the total prerogative of the atopic infant, its diagnostic value in older infants may also be limited.

The much better prognosis of infantile seborrhoeic dermatitis is confirmed by the findings of this study, where only three infants had a transient second episode of seborrhoeic dermatitis and infants were free of skin lesions by the age of 1 year. This was in contrast to the atopic group, where all infants had relapsed during the first year and at 1 year of age all had skin lesions ranging from mild in three infants to severe in four infants.

The apparent development of atopic dermatitis in infants initially diagnosed as having seborrhoeic dermatitis may result from the chance occurrence of seborrhoeic dermatitis in children with inherited atopic tendency (Dietal, 1965). However as a confident diagnosis on a single examination at an early age was difficult in many of the infants in our series, it may be that some of these reported cases may represent an error in the original diagnosis.

Thus from our clinical experience, apart from some guidance from the site of onset and distribution of the eruption, one has to adopt a 'wait and see' policy if the distinction between infantile seborrhoeic dermatitis and atopic dermatitis at presentation is difficult. Age of onset, family history and pruritus were found to be of limited diagnostic value in this series.

In a further study (Yates *et al.*, 1983) we have shown that total and antigen-specific IgE levels are of value in distinguishing the two conditions.

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Early diagnosis of infantile seborrhoeic dermatitis and atopic dermatitis—total and specific IgE levels

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SUMMARY

Total and antigen-specific IgE levels using the radioallergosorbent test (RAST) were measured in a prospective study of thirty-seven infants with dermatitis of early infancy, with the aim of distinguishing between atopic and infantile seborrhoeic dermatitis at an earlier age than is usually possible on clinical grounds. The results were correlated with the clinical diagnoses made after 1–2 years close follow-up. They showed that a RAST screen to egg white and to milk antibodies, and to a lesser extent total IgE levels, are of value in diagnosing atopic dermatitis at an early age and distinguishing this from infantile seborrhoeic dermatitis.

Raised serum IgE levels have been shown to precede the clinical manifestations of atopic disease (Polmar, Robinson & Minnefor, 1972; Hamburger *et al.*, 1974; Kjellman, 1976) and may therefore predict the later onset of atopic manifestations in individuals at risk. This may be of particular value in infancy (Orgel *et al.*, 1975), where the early diagnosis of atopic dermatitis is sometimes difficult. In a recent study (Yates, Kerr & MacKie, 1983), we demonstrated the difficulty of making a distinction on clinical grounds between atopic and infantile seborrhoeic dermatitis (including napkin psoriasis) in young infants. With the aim of distinguishing between these two conditions at an earlier age than is usually possible on clinical grounds, we have investigated the diagnostic and prognostic value of measuring both total and antigen-specific IgE levels in young infants suspected of having either atopic or infantile seborrhoeic dermatitis.

METHODS

We studied thirty-seven infants under 6 months of age who presented consecutively with a

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TABLE 1. Details of patients studied

No. of patients	Male	Female	Age at presentation		Follow-up time	
			Range	Mean	Range	Mean
37	23	14	3–24 weeks	14 weeks	14–30 months	19 months

clinical diagnosis of either atopic or infantile seborrhoeic dermatitis. Patient details are shown in Table 1. The clinical features and progress of these infants were closely followed during the first 1–2 years of life and during this period a diagnosis was reached purely on clinical grounds. At presentation (3–4 months), a 2–3 ml sample of blood was taken for estimation of total IgE levels, a RAST screen to seven allergens, IgA, IgM and IgG levels and an eosinophil count. These investigations were repeated at the ages of 6 months, 1 year and when appropriate 2 years. At the end of the study results were correlated with the clinical findings and diagnosis. Total IgE levels were measured in duplicate by a direct radioimmunosorbent technique (Ceskar & Lundkvist, 1972) using the Phadebas IgE PRIST kits (Pharmacia Diagnostics, Uppsala, Sweden). Results were expressed in U/ml of serum (1 U/ml = 1 kU/ml). The upper limit of normal was taken as the mean for the age of the infants plus two standard deviations. At the age of 3–4 months this was taken as 8 U/ml and at 1 year as 15 U/ml (Kjellman, Johansson & Roth, 1976). The specific IgE antibody activity was determined by a radioallergosorbent test (Wide, Bennich & Johansson, 1967).

Phadebas RAST kits with allergens linked to paper discs as the solid phase were used for the assays. Each serum sample was tested against a panel of seven allergens: egg white, milk, fish (cod), *Dermatophagoides pteronyssinus* (house dust mite), cat epithelium, dog epithelium and *Poa pratensis* (meadow grass pollen) and the results expressed in the Phadebas RAST score system: 0, 1, 2, 3 and 4, with 1 or more regarded as positive.

IgG, IgM and IgA levels were measured on serum samples by a single radial diffusion method (Fahey & McKelvey, 1965). Eosinophil counts were performed using a Fuchs-Rosenthal counting chamber. Eosinophilia was defined as more than 400 eosinophils/mm^3 and a high grade eosinophilia as more than 700 eosinophils/mm^3.

RESULTS

During the study period, nine infants were diagnosed on clinical grounds as having atopic dermatitis and twenty-seven as having infantile seborrhoeic dermatitis. In one child who had repeated episodes of erythroderma and a positive family history of both psoriasis and atopic dermatitis, a definite diagnosis has not yet been reached. Full clinical details of this group of infants have been reported elsewhere (Yates *et al.*, 1983).

Total IgE levels

Atopic patients. Total IgE levels at the ages of 3–4 months and 1 year are shown in Fig. 1. Eight out of nine infants with a clinical diagnosis of atopic dermatitis were found to have total IgE levels greater than 80 U/ml at 3–4 months and all nine had reached this level at 1 year. In four infants the total IgE titres were over 1000 U/ml.

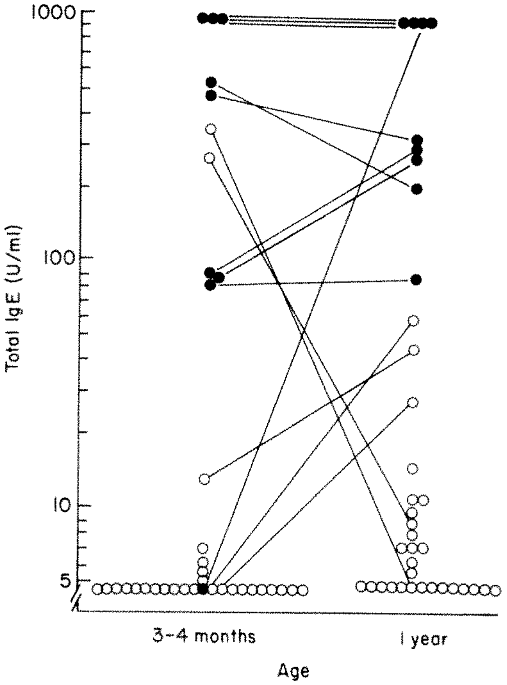

FIGURE 1. Total IgE levels in infants with atopic dermatitis (●——●) or infantile seborrhoeic dermatitis (○——○). Results are given for ages 3–4 months and 1 year.

Seborrhoeic dermatitis patients. Normal total IgE levels were recorded in twenty-four out of twenty-seven infants with seborrhoeic dermatitis at 3–4 months. Two children had markedly elevated levels (270 U/ml and 240 U/ml) and in a third child a value just above the mean + 2 s.d. for age was recorded (13 U/ml). The IgE levels of these infants were normal when repeated at 6 months and 1 year. However, at 1 year three other infants had IgE levels above the normal range (29 U/ml, 70 U/ml and 68 U/ml), the remaining twenty-four infants having normal IgE levels.

RAST results

Atopic patients. At 3–4 months all atopic infants had a RAST score of 1 or more to an average of four allergens (Table 2). The highest titres were to egg white and milk antibodies, and egg was positive in all of the atopic group and milk in seven out of nine atopic infants. Almost all other antibody titres recorded at this age were in the low range or RAST score 1 (Fig. 2).

By one year, nearly all of the atopic group had developed further IgE antibodies, notably to fish, animal danders and house dust mite. Those titres noted initially to be in the low range had risen significantly in almost all cases, the exception being to milk. Antibody levels to milk were declining in three infants, in only one of whom was cow's milk excluded from the diet. None of the infants had high pollen antibody titres at this age.

Seborrhoeic dermatitis infants. In contrast, twenty-one out of twenty-seven infants had a completely negative RAST screen at 3–4 months and twenty-three out of twenty-seven at 1 year. Those positive antibody titres recorded were all in the low range or RAST score 1 at both

TABLE 2. Numbers of positive RAST tests found when sera of infants with atopic dermatitis and infants with infantile seborrhoeic dermatitis were tested against a panel of seven allergens by the RAST technique

No. of allergens positive on RAST screen	Atopic dermatitis (n = 9)		Infantile seborrhoeic dermatitis (n = 27)	
	3–4 months	1 year	3–4 months	1 year
All negative	0	0	21 (78%)	23 (88%)
1	1	0	4	1
2	1	1	2	3
3	1	1	0	0
4	1	1	0	0
Over 4	5 (56%)	6 (67%)	0	0

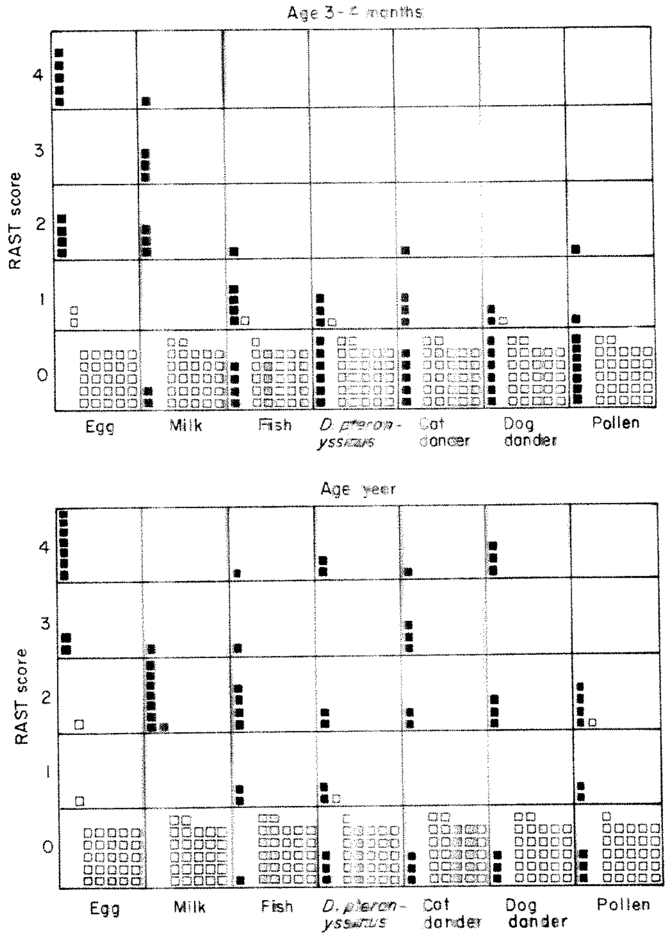

FIGURE 2. Comparison of antigen-specific IgE levels (RAST technique) to seven allergens in infants with atopic dermatitis (■) or infantile seborrhoeic dermatitis (□). Results are given at ages 3–4 months and at 1 year.

3–4 months and 1 year, apart from three individuals in whom RAST scores of 2 were recorded (Fig. 2). Only two individuals at 3–4 months and three at 1 year, had more than one antibody recorded in the RAST screen and unlike the atopic infants, none of the group developed further IgE antibodies at 1 year, nor did any established titres rise.

It can be seen that there are clear differences between RAST screens in the seborrhoeic and atopic infants. If antibody titres in the low range or RAST score 1 are excluded as being of doubtful significance (Wraith *et al.*, 1979), 98% of seborrhoeic infants at 3–4 months and 92% at 1 year had normal RAST screens, whereas all of the atopic infants had abnormal RAST screens at both ages. Furthermore this applies if only egg white and milk antibodies are considered.

The remaining infant in whom a definite clinical diagnosis has not yet been reached, had an antigen-specific IgE level of RAST score 1 to egg white at 3–4 months and to egg white and milk antibodies at 1 year. His total IgE levels were normal at 3–4 months and 1 year.

Eosinophil counts

Eight out of nine (88%) infants with atopic dermatitis had a high grade eosinophilia noted on at least one occasion. However counts were very variable for each individual, even on a day to day basis. Three of the atopic group had normal eosinophil counts recorded when IgE levels were over 1000 U/ml. Eleven patients (40%) with seborrhoeic dermatitis had eosinophilia noted on at least one occasion and more than half of these were of high grade. The overall positive correlation between IgE levels and eosinophil counts (i.e. elevated IgE levels with eosinophilia and normal IgE levels with normal eosinophil count) was 75%.

Immunoglobulin levels

No significant differences were observed in serum concentrations of immunoglobulins A, G and M between infants with atopic dermatitis and those with seborrhoeic dermatitis. Figure 3 shows

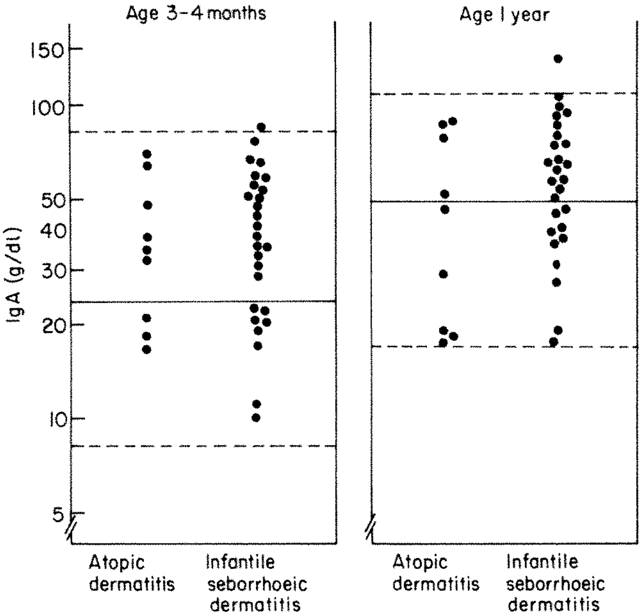

FIGURE 3. Comparison of IgA levels measured in infants with atopic dermatitis or infantile seborrhoeic dermatitis at 3–4 months and 1 year. Mean ± 2 s.d. values for healthy infants aged 3–4 months and 1 year as reported by Hobbs (1970) are shown.

the results of IgA measurements made in the two groups of infants at the ages of 3–4 months and 1 year. No transient dip in IgE levels was recorded in any member of the atopic group.

DISCUSSION

This study shows that total IgE levels are of value in the early differentiation between infantile seborrhoeic dermatitis (including napkin psoriasis) and atopic dermatitis. Measurements of antigen-specific IgE levels would appear to be of even greater value. About 5% of atopic patients have been reported to have normal serum IgE levels (Orgel et al., 1975). However, some patients may be exquisitely sensitive to only one allergen and total IgE levels may be low while RAST and prick tests are positive to only one or two specific allergens. The atopic infant in whom a normal IgE level was recorded at 3–4 months, had raised specific IgE titres to milk, cat epithelium and pollen at this age. Furthermore, the two seborrhoeic infants in whom high total IgE levels were found at 3–4 months had normal RAST screens. Scabies infestation and parasites in the stool were both excluded as a cause of raised IgE antibodies. Although it is possible that we might have missed relevant allergens in our RAST screen, both IgE levels were normal when repeated at 6 months and 1 year. IgE titres of 70 U/ml and 68 U/ml were recorded in two infants at 1 year who were clinically thought to have infantile seborrhoeic dermatitis. These raised total IgE levels were associated with RAST scores of 1 to pollen and egg white respectively. At the age of 18 months neither infant has any manifestations of atopy and comparison of their allergy patterns with those of the atopic group shows differences both in titres and numbers of antibodies recorded.

Total IgE levels at 1 year correlated well with disease activity in that those atopic infants with IgE levels over 1000 U/ml had the most active eczema and those with lower but still raised IgE levels had less severe eczema.

The pattern of positive RAST tests in the young atopic infants changed with increasing age. At 3–4 months the most commonly found IgE antibodies were to egg white and milk. Titres to egg white tended to rise during the first year or to be maintained at already high levels. Clinical evidence of egg allergy was found in only one infant, who developed a facial rash after eating eggs. However it seems that cases of egg-positive RAST results in egg-tolerant patients are not unusual (Schur, Hyde & Wypych, 1974; Dannaeus et al., 1977). In contrast to egg white titres, in some infants IgE titres to milk were declining by the age of 1 year, as has been recorded by other workers (Foucard, 1973; Hannukesla, 1980). In one infant this may have been helped by exclusion of cow's milk from the diet. Two atopic infants who were exclusively breast-fed at the time of onset of their skin disease had no detectable antibodies to cow's milk at 3–4 months of age, although both developed antibodies to milk and other proteins at a later date. Fish (cod) was the only other food allergen measured and antibody titres rose with age and were present in eight out of nine patients at one year of age. This presumably correlates with the introduction of fish into the diet.

In contrast to food allergens, IgE antibodies to animal danders and house dust mite were either not detectable or only occurred in low titres at 3–4 months. However, titres had reached very high levels in over half the infants by 1 year. Comparable IgE anti-pollen titres have so far not been detected. Foucard (1973) found that animal danders were not detected until 18 months of age and those to pollen until 3 years of age. Our findings are similar although we have found antibodies to animal danders at a slightly earlier age.

Infant allergy patterns, particularly in the first few months of life, differ from those found in the adult atopic. MacKie et al. (1979) found positive specific IgE antibodies to *D. pteronyssinus*, pollen and cat epithelium by both RAST and prick tests in all their adults with atopic eczema, and suggested that if IgE antibody testing was used as a diagnostic screen for atopy, positive results to these three allergens would be found in all atopic subjects. Other workers (Merrett & Merrett, 1978; Wraith *et al.*, 1979) have used the same antibody screen to establish the atopic status of patients. This screen would not detect atopy in the majority of young infants and from our findings it would appear that a RAST screen to egg white, milk and possibly fish would be of more value in this age group.

In view of the wide variation found in eosinophil counts in each infant and the presence of eosinophilia in a significant proportion of patients with seborrhoeic dermatitis, we feel that these are of little value in the differentiation of atopic and seborrhoeic dermatitis. This is in contrast to Foucard (1974) who showed that eosinophil counts were of value in predicting the onset of asthma in patients with wheezy bronchitis.

In conclusion, clinical distinction between infantile seborrhoeic and atopic dermatitis is difficult in the young infant, but our findings suggest that a RAST screen to egg white, cow's milk and possibly fish, and to a lesser extent total IgE levels, will aid in their differentiation as early as 3–4 months of age. Our results also support the view that atopic dermatitis and infantile seborrhoeic dermatitis are distinct entities, which is borne out by their very different prognoses.

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Metabolic changes at the margin of the spreading psoriatic lesion

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SUMMARY

Keratotome slices were cut across the margins of rapidly-spreading psoriatic plaques. Each slice was divided into eight sections and in each section we measured the percentage cells in S phase and the levels of glucose-6-phosphate dehydrogenase (both related to epidermal proliferation), acid phosphatase (associated with keratinization) and alkaline phosphatase (a marker for dermal capillaries).

Disturbances in the epidermis extended only 2 to 4 mm into the 'uninvolved' skin, whereas the capillaries were metabolically abnormal for a distance of about 2 cm ahead of the advancing edge of the plaque. This implies that changes in the capillary may precede those in the epidermis during the spread of the psoriatic lesion.

The psoriatic lesion is characterized by three signs: thickening, scaling and erythema. At the cellular level, these correspond to abnormalities in three more or less distinct regions of the skin: increased proliferation of the lower epidermis, incomplete keratinization (often with loss of the granular layer and parakeratosis) in the upper epidermis, and tortuous, distended capillaries in the dermal papillae. In each case the morphological changes are accompanied by characteristic alterations in the metabolic patterns of the tissues. However, the relationship between these phenomena remains speculative. Each of the three has, at one time or another, been nominated as the 'primary' expression of psoriasis (Mier & Cotton, 1976).

A popular experimental approach to this problem is the study of the margin of a spreading psoriatic plaque, but different authors are by no means in agreement as to the sequence in which the various changes occur. Surprisingly, most of this work has been carried out employing histological or histochemical observation as the sole criterion of 'abnormality'. The use of more objective measurements coupled with statistical evaluation of data might provide a more clear-cut answer.

In this investigation we have studied the following events at the margins of spreading lesions:

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(i) Cell cycle kinetics (percentage of cells in S phase) and glucose-6-phosphate dehydrogenase (G6PDH) activity. G6PDH occurs chiefly in the proliferative zone of the epidermis and is grossly elevated in the psoriatic lesion (Halprin & Ohkawara, 1966).

(ii) Acid phosphatase (ACP). This is found mainly in the upper epidermis (Mier *et al.*, 1976) and is probably associated with the keratinization process. It is increased in the psoriatic plaque (Mier & van den Hurk, 1976).

(iii) Alkaline phosphatase (ALP). This is a well-established marker for the capillaries (Fisher & Glick, 1947; Kopf, 1957; Pirilä & Eränkö, 1950). Biochemical assay has shown it to be almost exclusively dermal (Mier & van Rennes, 1982b) and to be increased about 4-fold in the psoriatic lesion (van de Kerkhof *et al.*, 1982).

MATERIALS AND METHODS

Materials

Glucose-6-phosphate and NADP were obtained from the Sigma Chemical Co. (St Louis, U.S.A. and 4′,6-diamidino-2-phenylindole 2 HCL (DAPI) from Serva Feinbiochemica GmbH (Heidelberg, Germany). The sources of reagents employed for ACP and ALP assay have been described previously (Mier & van den Hurk, 1975; Mier & van Rennes, 1982b).

Subjects and methods of biopsy

Patients with unstable progressive plaque psoriasis were employed for this study. No therapy (local or systemic) had been used for at least 2 weeks before biopsy. Control subjects were paid volunteers with no history of skin disease.

A growing lesion (5–10 cm in diameter) on the back or upper arm of three patients was selected for investigation, and the edge was marked with a fine-tipped felt pen. Care was taken to ensure that the skin outside this boundary appeared absolutely normal by macroscopical inspection. After cooling the skin surface with an ethyl chloride spray, a slice of 8 × 16 mm was cut across the margin of the plaque (Fig. 1) using a Castroviejo keratotome set for a depth of 0·2 mm in conjunction with a metal 'guard'.

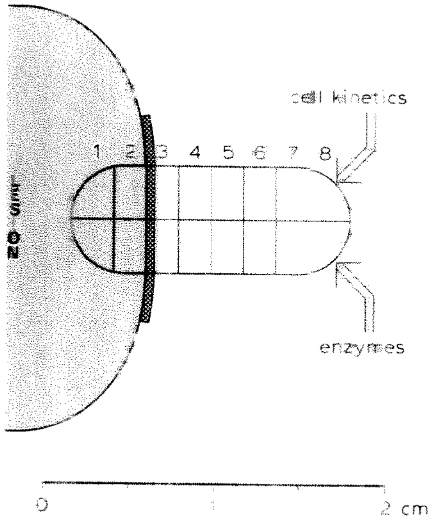

FIGURE 1. Scheme showing position and method of subdivision of keratotome slice.

The biopsy was spread on to filter paper moistened with phosphate-buffered saline (PBS) and dissected freehand into eight consecutive sections, of which two were from the lesion and six from the clinically normal skin. Each section was then subdivided into two portions (Fig. 1). The smaller portions were placed in ice-cold PBS for cell-cycle analysis and the larger ones were weighed and stored at −70°C before homogenization for biochemical analysis.

Microbiopsies were cut freehand from the clinically uninvolved skin of six additional psoriatic patients at exactly 2 and 3 cm from the edge of a lesion, and from the back or upper arm of eleven control subjects, using a razorblade in conjunction with a metal guard with a hole 4 mm in diameter. No anaesthetic agent was employed. These samples were weighed and stored at −70°C for enzymatic analysis.

Cell-cycle analysis

This was performed as described by Bauer *et al.* (1980). Briefly, cells were isolated by trypsinization in the presence of dithioerythritol and stained with propidium iodide. DNA histograms were prepared using an ICP 11 impulse cytophotometer (Phywé, Germany). The percentage of cells in S phase was calculated by an on-line computer system (Hewlett Packard 9810 A). All samples were processed on the day of biopsy.

Preparation of extracts

The second portion of each section of the psoriatic slices and the intact microbiopsies were homogenized in 1 ml of 1 mg/ml aqueous bovine serum albumin solution (BSA), using an all-glass Potter type homogenizer fitted with an ice jacket. The homogenate was centrifuged (1000 ***g***, 10 min) to yield a clear supernatant ('extract') and the residue was discarded. Samples were processed within 1 week of biopsy and the subsequent assays performed immediately after homogenization.

Biochemical measurements

The DNA concentrations of the extracts were determined as the fluorescent complex with DAPI according to Kapuścińsky & Skoczylas (1977) with modifications described by Mier & van Rennes (1982a).

G6PDH was assayed by fluorescence measurement of the NADPH released during the reaction of glucose-6-phosphate with NADP. Duplicate 20 μl aliquots of the extract were incubated (30 min, 37°C) with 100 μl of a reagent containing 50 mmol/l tris (pH 7·6), 10 mmol/l $MgCl_2$, 6 mmol/l EDTA, 0·2 mmol/l glucose-6-phosphate and 0·1 mmol/l NADP. The reaction was stopped by the addition of 500 μl of ice-cold carbonate-bicarbonate buffer (0·2 mol/l, pH 10·5) and the NADPH determined by fluorescence. Preliminary experiments were carried out to verify linearity with incubation time and tissue concentration over the ranges employed. G6PDH activity was calculated as nmol NADPH released per min per μg DNA.

ACP was determined by hydrolysis of 4-methylumbelliferyl phosphate at pH 3·6 as described previously (Mier & van den Hurk, 1975) but using 20 μl aliquots instead of 100 μl as originally reported. Enzyme activity was calculated as nmol 4-MU released per min per μg DNA.

ALP was assayed by hydrolysis of 4-methylumbelliferyl phosphate at pH 9·8 in the presence of 5 mmol/l NaF as described by Mier & van Rennes (1982b). Activity was expressed as pmol 4-MU released per min per mg fresh weight of tissue.

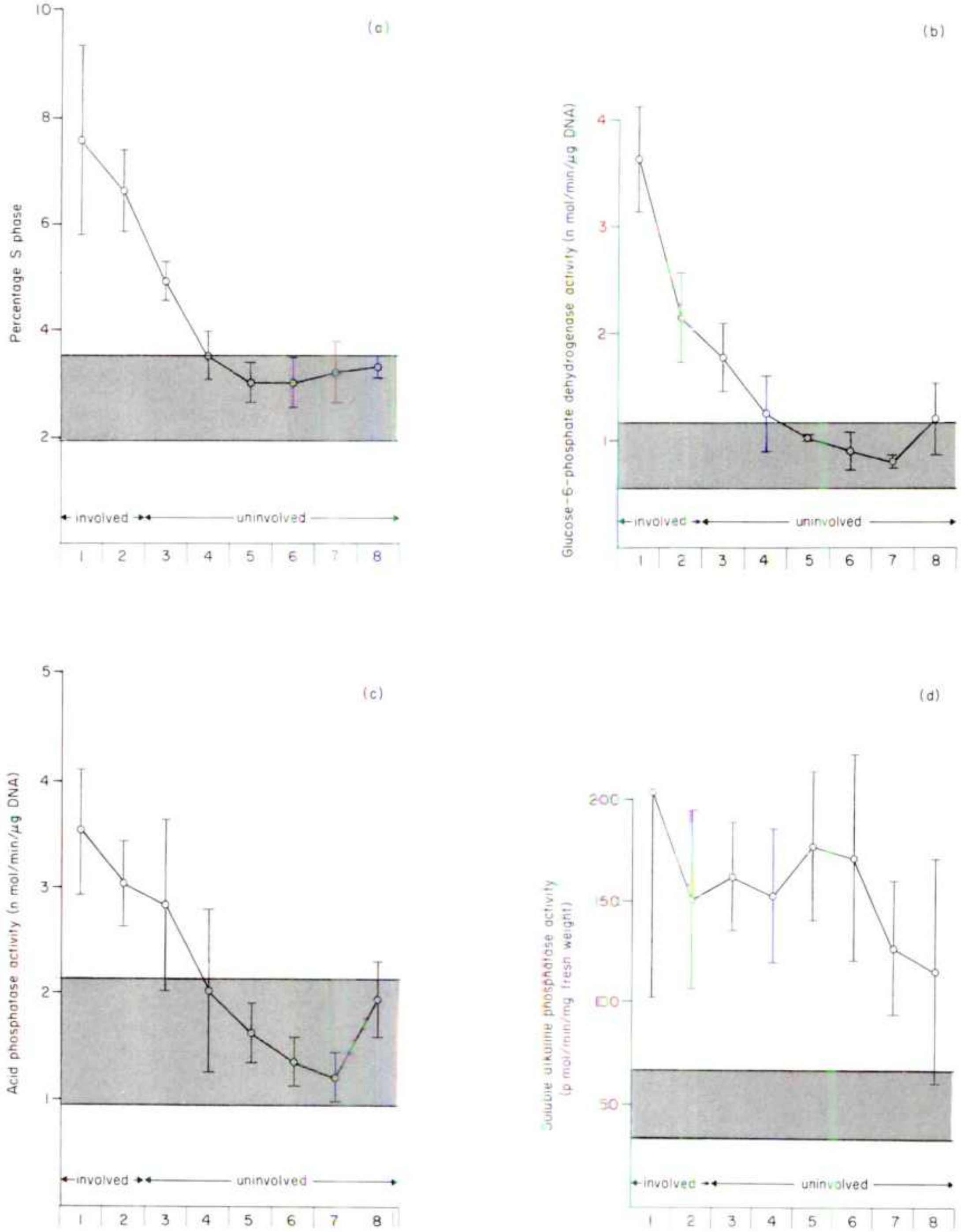

FIGURE 2. Mean values (± s.e.m.) of: (a) percentage S phase, (b) G6PDH, (c) ACP and (d) ALP. Numbers on the horizontal axis correspond to the sections illustrated in Fig. 1, and the shaded areas indicate normal ranges.

RESULTS

Normal ranges

The shaded areas in Fig. 2a–d represent the mean ± standard deviation for each measurement in biopsies from healthy controls. Values for percentage S phase (2·7 ± 0·8, n = 60) are taken from Bauer *et al.* (1980). Ranges for G6PDH and ACP (0·87 ± 0·31 and 1·56 ± 0·59 nmol/min/μg DNA respectively) were established during this investigation using eleven control specimens; for comparison with earlier literature these have also been calculated on a fresh weight basis, yielding mean values of 0·95 and 1·72 nmol/min/mg respectively. The normal range for ALP (51 ± 16 pmol/min/mg fresh weight, n = 24) includes thirteen specimens reported previously (van de Kerkhof *et al.*, 1982) plus eleven additional specimens.

Psoriatic biopsies

Each point in Fig. 2a–d represents the mean ± standard error of the mean of three independent experiments. It is seen that the values of all four variates for sections 1 and 2 (i.e. those falling within the lesion) are markedly elevated, the increases ranging from a little over 2-fold for ACP to about 4-fold for G-6-PDH and ALP.

The epidermal variates (percentage S phase, G-6-PDH and ACP) follow a very similar course outside the lesion, all entering the normal range at about section 4, i.e. 2–4 mm from the edge of the clinically involved skin. The behaviour of the capillary marker, ALP, is strikingly different; it is seen from Fig. 2d that the ALP level is still distinctly elevated more than 1 cm away from the lesion. Microbiopsies taken 2 cm from the lesion indicate ALP levels to be in the upper part of the normal range (61 ± 11 pmol/min/mg, n = 6). At a distance of 3 cm, however, the activity is no longer elevated (44 ± 5 pmol/min/mg, n = 6).

DISCUSSION

Both the normal ranges established here and also the values for the plaque (sections 1 and 2) are in reasonable agreement with earlier literature. Although no measurements have been made on the 'distant' uninvolved skin during the present investigation, previous workers have established that all four variates in the uninvolved skin are essentially normal (Bauer *et al.*, 1981; Halprin & Ohkawara, 1966; Mier & van den Hurk, 1976; van de Kerkhof *et al.*, 1982).

Our data for the sections outside but immediately adjacent to the lesion make it clear that metabolic disturbances associated with the dermal capillaries extend to a much greater distance than those associated with the epidermis. Since we have selected patients whose lesions were in a rapidly growing phase, we may reasonably conclude that the dermal changes in fact occur before those in the epidermis. This is in agreement with the views of Civatte (1924) and Pinkus & Mehregan (1966) but is in conflict with other workers, including Christophers & Braun-Falco (1970).

It is tempting to assume that temporal primacy implies causality, and to conclude that the 'cause' of psoriasis must be sought in the dermis. However, although our findings are certainly compatible with such a hypothesis (for example, Cotton & Mier, 1964), other explanations are by no means excluded. Further approaches which may throw light on this question include the provocation of lesions in previously uninvolved psoriatic skin and the time course of biochemical events during therapeutic clearance of established plaques.

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Ultrastructure of pityriasis rubra pilaris with observations during retinoid (etretinate) treatment

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SUMMARY

The light and electron microscopic structure of pityriasis rubra pilaris (PRP) is described in five patients. Hyperkeratosis, hypergranulosis, keratotic plugs in the follicular openings, acanthosis and focal parakeratosis were observed. A moderate perivascular infiltrate was seen in the upper dermis. Electron microscopy revealed moderately activated keratinocytes, a decreased number of tonofilaments and desmosomes, enlarged intercellular spaces, parakeratosis with lipid-like vacuoles and a large number of keratinosomes. Lymphoid cells were present in the epidermis in moderate numbers. At the dermo-epidermal junction, the basal lamina was focally split, containing gaps.

Etretinate therapy produced moderate to marked clinical improvement. The histological picture improved but the typical signs of PRP, including follicular plugging, persisted. Ultrastructurally the cellular activity and the amount of hyperkeratosis and parakeratosis decreased, while increases in keratinosomes, intercellular substance, microvilli and desmosomes were observed during treatment.

Pityriasis rubra pilaris (PRP) is an uncommon cutaneous disease characterized by keratotic follicular papules, palmo-plantar hyperkeratosis and bright red erythroderma with scattered islands of normal skin (Griffiths, 1976, 1980; Niemi *et al.*, 1976). The disease is subclassified into five types including both hereditary and acquired forms (Griffiths, 1980). A detailed histological description of PRP has been presented (Niemi *et al.*, 1976), but the ultrastructure has not been described. The role of vitamin A deficiency as an aetiological factor has been the subject of considerable study, and etretinate has been tried as a treatment for PRP. Moderate to good

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results have been obtained in our clinic (Lauharanta & Lassus, 1980) and elsewhere (Peck *et al.*, 1978; Marks, Finlay & Holt, 1981).

In the present paper, we describe in detail the fine structure of the skin in PRP and report findings during treatment with etretinate. Some of the results have been presented at recent conferences (Kanerva *et al.*, 1982a,b).

METHODS

Five patients with PRP were studied. Details of the patients and timing of biopsies are presented in Table 1. The patients were given etretinate at an initial dose of 0·75–1·00 mg/kg per day. The dose was later reduced to a maintenance level of 0·50–0·90 mg/kg according to the clinical response.

Biopsies were taken for light and electron microscopy (Table 1). For ultrahistopathology, 1 mm^3 or smaller blocks were fixed in cacodylate or phosphate-buffered (0·1 M; pH 7·3) 2·5% glutaraldehyde (osmolality 530 mOsm/kg H_2O) at 4° C and postfixed with 1% osmium tetroxide and processed as described previously (Kanerva *et al.*, 1981, 1982c,d). The morphological calculations presented in Table 2 were semiquantitative. The vitamin A level, the retinol-binding protein (RBP) and the pre-albumin level in serum were measured before treatment and during treatment in three patients by the methods described previously (Lauharanta, 1981).

RESULTS

Clinical aspects

Patients 1 and 5 were initially treated with etretinate, which had a definite therapeutic effect in both cases within 4 weeks. In patient 1 the treatment could be discontinued later with no relapse, while in patient 5 treatment has been continued so far for 3 years with a maintenance dose of 0·3 mg/kg per day with almost complete remission of the symptoms. In patient 2, the treament was terminated after 4 months since only partial improvement occurred and the patient complained of diffuse alopecia and cheilitis. Patient 3 has been treated for 6 months and patient 4 has been treated for 8 weeks, so far with a favourable clinical result.

The serum vitamin A, retinol-binding protein and pre-albumin were studied in three patients before and during treatment. The mean (± s.e.m.) levels before treatment were within normal

TABLE 1. Details of patients with PRP and timing of biopsies

Patient	Type of PRP (Griffiths, 1976)	Sex	Age	Duration of PRP	Timing of biopsies during treatment
1 (M.T.)	I	M	55	8 months	o——o—o——o————o
2 (R.M.)	I	M	31	6 years	o——o————o———
3 (J.V.)	I	M	23	2 weeks	o————————
4 (S.R.)	I	M	40	6 months	o—o—o—o——o
5 (J.L.)	III	M	18	15 years	o——o—o—o—o—o—o—o
					⊢—PUVA—⊣
					0 1 2 4 6 8 10 12 14
					week of retinoid treatment

limits, i.e. 1·5 ± 0·3 μmol/l (vitamin A), 44 ± 6 mg/l (retinol-binding protein) and 250 ± 28 mg/l (pre-albumin) and remained practically unchanged during treatment.

Patients 1 and 4 had been previously, and patient 5 was currently, treated with PUVA, which resulted in deterioration.

Light microscopy

The light and electron microscopic findings are summarized in Table 2.

TABLE 2. Summary of light and electron microscopic observations of pityriasis rubra pilaris (PRP) (five patients) before and during etretinate therapy compared with normal epidermis of controls. + + + = large size, (number or activity), + + = moderate, + = small, ± = very small, − = absent

	Untreated PRP	Etretinate therapy of PRP 2–4 wk	Etretinate therapy of PRP 6–14 wk	Normal skin
EPIDERMIS				
Stratum malpighii				
Keratinocytes				
Polysomes and free ribosomes	+ +	+ +	+(+)	+
Mitochondria	+ +	+ +	+(+)	+
Rough endoplasmic reticulum	+ +	+ +	+(+)	+
Golgi apparatus, size and number	+ +	+ +	+(+)	+
Microvilli, number	+	+ +	+ +	±
Mitoses, number	+(+)	+(+)	+	+
Intercellular space:				
width	+ +	+ + +	+ +	+
amorphous or granular material	+ +	+ + +	+ +	±
Perinuclear vacuoles	+ +	+ +	+(+)	−
Tonofilaments, number	+	+	+ +	+ + +
Desmosomes, size and number	+	+	+ +	+ + +
Cell size	+ +	+ +	+ +	+
Nucleoli, size	+ +	+ +	+ +	+
Lysosomes, number	+	+	+	+
Keratohyalin (KH) cell layers with KH	+ +	+ +	+ +	+
Keratinosomes and keratinosome-derived lamellae	+ +	+ + +	+ +	+
Gap junctions	+	+	+	+
Necrotic keratinocytes	±	±	±	±
Langerhans cells, number	+	+ +	+ +	+
Lymphoid cells	+ +	+ +	+	−
Neutrophils	−	−	−	−
Stratum corneum				
Cell layers	+ +	+(+)	+(+)	+
Lipid droplets	+ + +	+ + +	+(+)	−
Parakeratosis	+ +	+ +	+(+)	−
Transitional cells	+ +	+ +	+	−
PAPILLARY DERMIS				
Capillaries, size	+ +	+ +	+(+)	+
Pericapillary cell infiltrate	+ +	+ +	+(+)	−

By light microscopy the typical features of PRP (Niemi *et al.*, 1976) were seen in all five patients before treatment: dense hyperkeratosis, keratotic plugs in the follicular openings with parakeratosis on the shoulders, irregular acanthosis and a moderate perivascular infiltrate in the upper dermis (Fig. 1). The stratum granulosum comprised 3–5 cell layers. The granular cells were often swollen and enlarged.

During the initial 2–4 weeks of etretinate treatment, the hyperkeratosis was reduced (Fig. 2), but all the other histological signs of PRP were present (Figs 1 and 2). Focally the morphology was totally unchanged (Fig. 1) despite an apparent clinical improvement. During further therapy for up to 14 weeks, the histological picture slightly improved, although all the typical signs persisted, including hyperkeratosis and follicular plugging.

Patient 5 was treated with PUVA after 4 weeks of etretinate. This led to a clinical and histological activation of the PRP signs. At 6 weeks of PUVA an inflammatory reaction occurred with migration of polymorphonuclear leukocytes into the epidermis and PUVA treatment was discontinued.

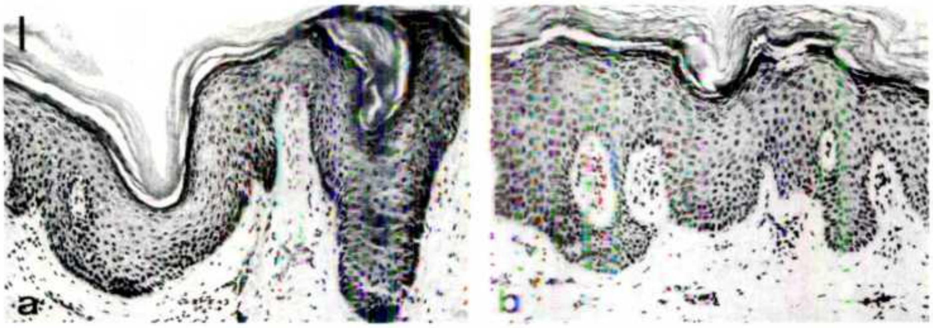

FIGURE 1. Light micrograph of patient 5, (a) before treatment and (b) after 4 weeks of etretinate treatment. No histological improvement is seen.

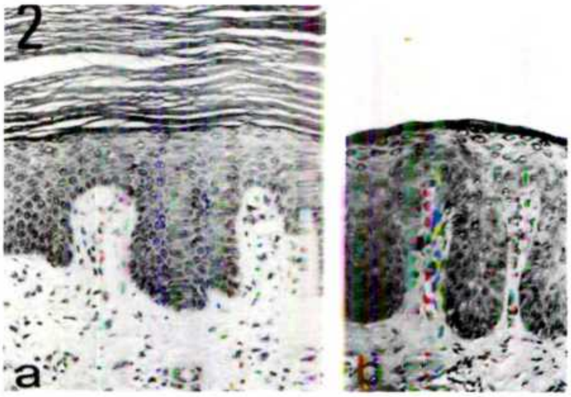

FIGURE 2. Light micrograph of patient 4 (a) before treatment and (b) after 4 weeks of etretinate treatment. A clear decrease of hyperkeratosis is seen.

Electron microscopy

Untreated PRP

Dermo-epidermal junction, basal and prickle cells. The hemi-desmosomes and anchoring fibrils were normal. The basal lamina was sometimes split. Lymphocytic cells and keratinocyte processes were seen to protrude through occasional basal lamina gaps. The basal cells and prickle cells showed a slight to moderate increase of cellular activity. The cells contained large numbers of ribosomes and polysomes (Fig. 3a) and also increased Golgi areas, mitochondria and endoplasmic reticulum. The tonofilaments were decreased compared with normal, as was the

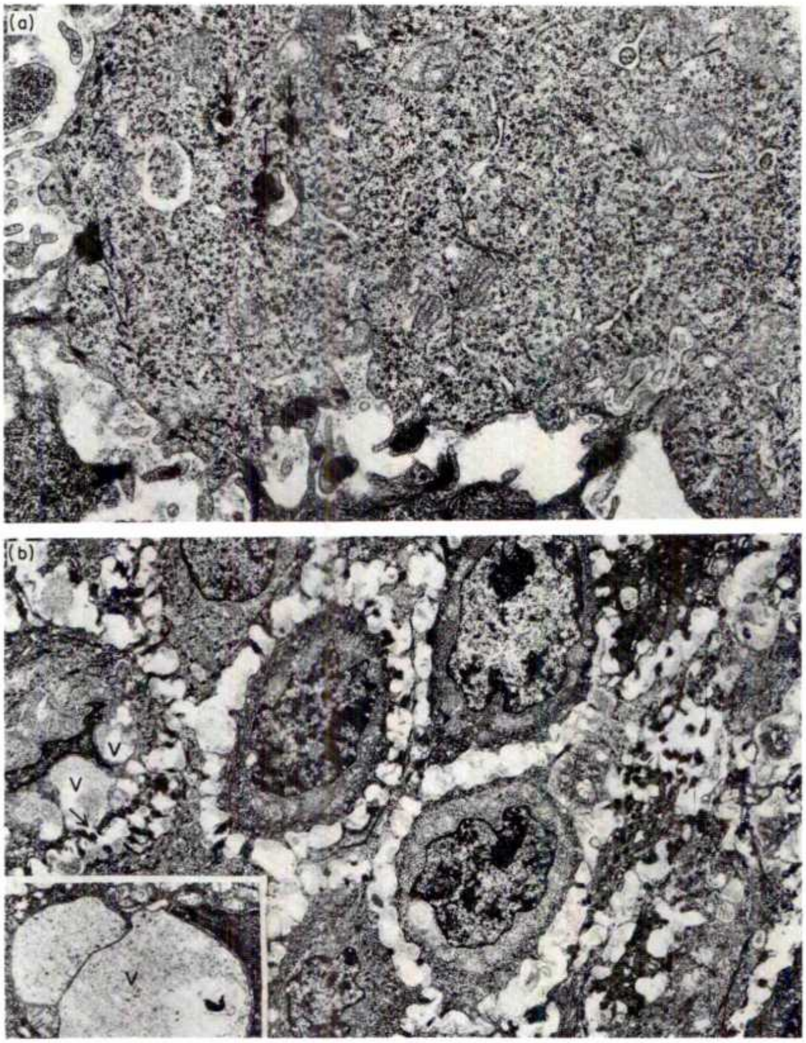

FIGURE 3. Untreated PRP. (a) Prickle cell with scant tonofibrils and abundant polysomes. Arrows, intracellular desmosome (× 15,120). (b) Prickle cell layer with enlarged intercellular spaces. Vacuolated (v) prickle cells are seen in the inset (× 6370) and at the left. Connection between vacuole and the intercellular space is marked by an arrow (× 4760).

number of desmosomes (Fig. 3a,b). The prickle cells were often large and rounded. Mitotic figures were observed on occasion. The intercellular space was focally clearly enlarged (Fig. 3b) containing an amorphous ground substance with small to large amounts of granules, which were morphologically identical to intracellular ribosomes (Fig. 4). The perinuclear area was often free from tonofibrils containing mainly ribosomes and mitochondria (Fig. 3b). Vacuoles (Fig. 3, 4), sometimes with connections to the intercellular space (Fig. 3b, 4a), were frequent in the prickle cells. Microvilli were numerous.

Stratum granulosum. The number of apposed processes containing keratohyalin was increased up to nine cell layers (Fig. 5a). Keratohyalin was morphologically normal. Keratinosomes and keratinosome-derived lamellae in the intercellular space were frequent in the transition zone. Transitional cells with a marginal band were seen regularly (Fig. 5a,b).

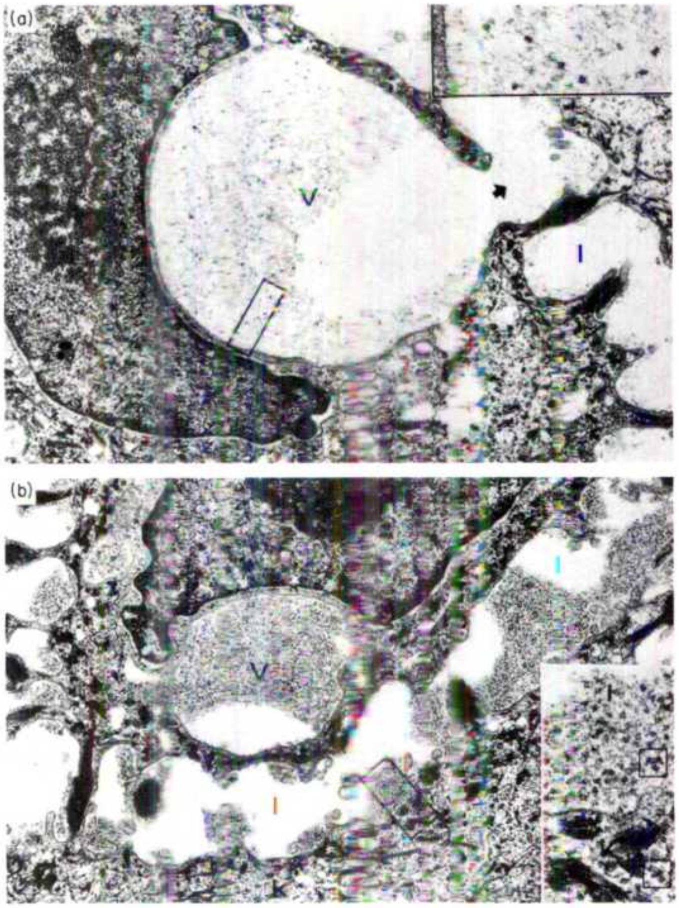

FIGURE 4. Untreated PRP. Vacuolated (v) prickle cells and intercellular substance (I) at greater magnification. Note the moderate (a) to large (b) amount of intercellular and vacuolar substance. The substance is comprised of moderate (a, inset) to large (b, inset) amounts of granules identical to intracytoplasmic ribosomes (rectangles), (×15,000). Insets (×36,000).

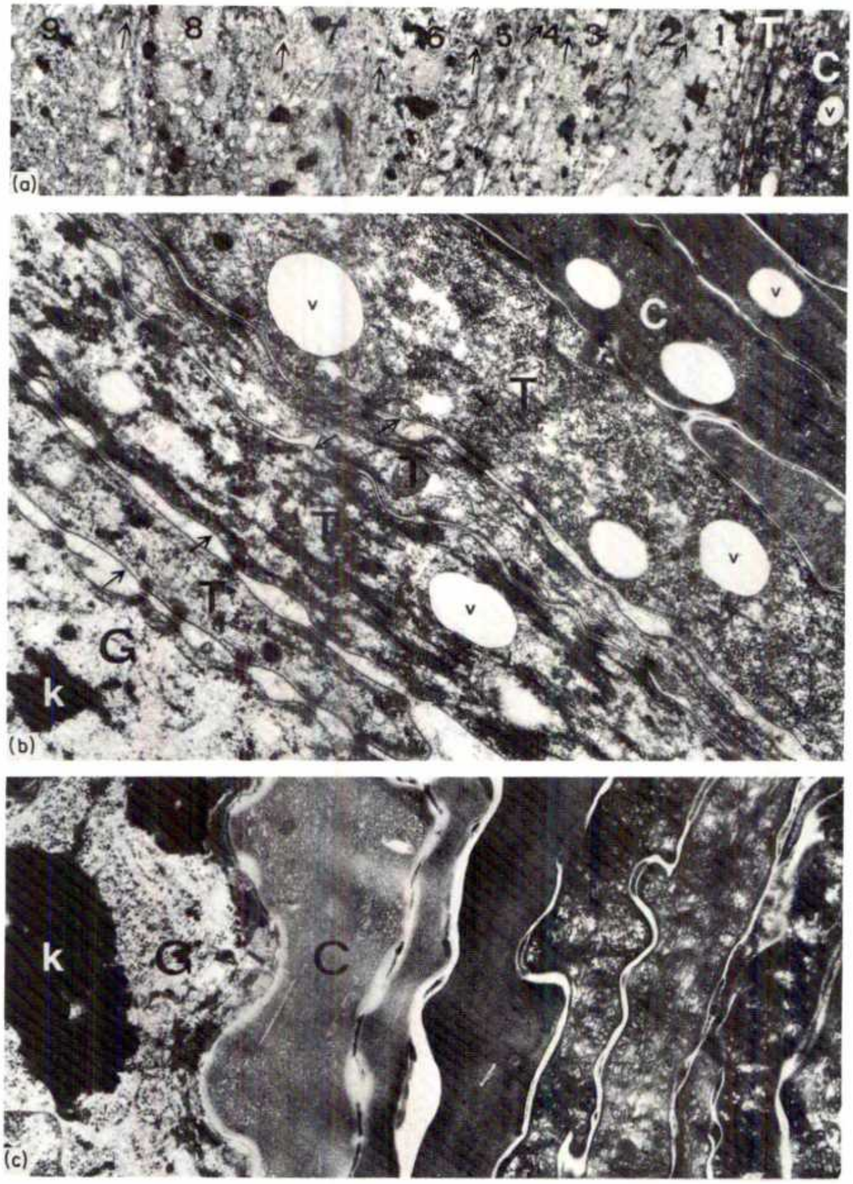

FIGURE 5. Untreated PRP. (a) Flattened processes from nine keratinocytes (1–9) with keratohyalin. Arrows in the intercellular spaces indicate desmosomes. T = transitional cell; C = corneocyte; v = lipid-like vacuole (×4050). (b) Four transitional cells (T) with prominent marginal band (arrow) between stratum granulosum (G) with keratohyalin (k) and stratum corneum (C). Note the abundant lipid-like vacuoles (v), (×19,500). (c) 8 weeks of retinoid treatment of PRP. Note the abrupt change from stratum granulosum (G) with keratohyalin (k) to stratum corneum (C) as compared to Figs 5a,b (before treatment), (×24,750).

Stratum corneum. In addition to transitional cells, parakeratotic cells with pyknotic nuclei were frequent. The lowermost horny cells were compact, exhibiting a keratin pattern with low density filaments set in an amorphous substance (Fig. 5b), while the upper cells had a more fibrillar appearance. Desmosome discs, intercellular substance and the marginal band were normal. Lipid droplet-like vacuoles were frequent in the transitional cells and in the corneocytes (Fig. 5a,b).

Non-keratinocytes. Melanocytes were normal in number and appearance. Lymphoid cells were abundant in the epidermis. They were either solitary or formed small clusters of 3–5 cells. Both macrophages and small lymphocytes were observed. Langerhans cells appeared normal and were sometimes apposed to lymphoid cells (Fig. 6).

Papillary dermis. The blood capillaries were moderately widened in the papillary dermis. They were usually unfenestrated, although the endothelial wall was thinned compared to normal. Fenestrated capillaries were also observed. A moderate pericapillary lymphoid cell infiltrate was observed.

The effect of retinoid treatment

Compared with untreated PRP, the number of cell organelles (ribosomes, mitochondria, endoplasmic reticulum, and Golgi areas) per keratinocyte decreased, indicating decreased cell activity. At the dermo-epidermal junction, a split basal lamina was still often found after 6–8 weeks of treatment. The intercellular space remained enlarged during the first 6 weeks. The amount of intercellular substance even increased in three patients during 2–6 weeks of therapy. After 14 weeks of treatment, the desmosomes were still small in size and number and the intercellular spaces were large containing a granular substance. The number of microvilli further increased (Fig. 7a) and remained increased during therapy. Keratohyalin was not influenced, but the keratinosomes and keratinosome-derived lamellae in the intercellular space of the transition zone were increased (Fig. 7b). The number of horny cell layers decreased slightly during treatment as did the amount of lipid-like droplets and parakeratotic cells of the horny layer (Fig. 5c). However, both lipid droplets and parakeratosis were regularly observed at

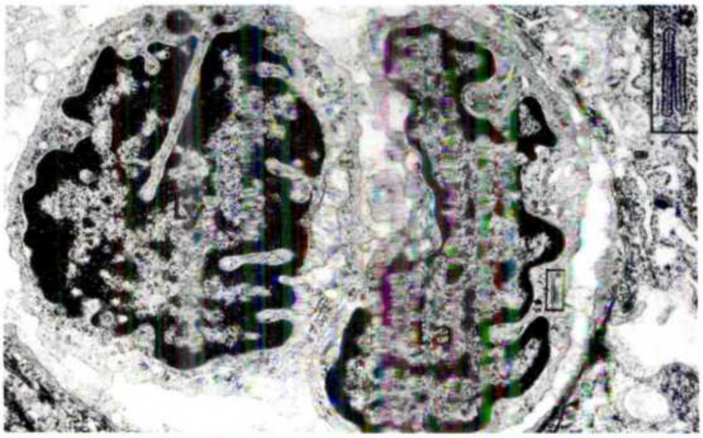

FIGURE 6. Untreated PRP. Langerhans cell (La) apposed to lymphocyte (Ly) in upper prickle cell layer. Inset: Langerhans granule (× 21,000). Inset (× 30,000).

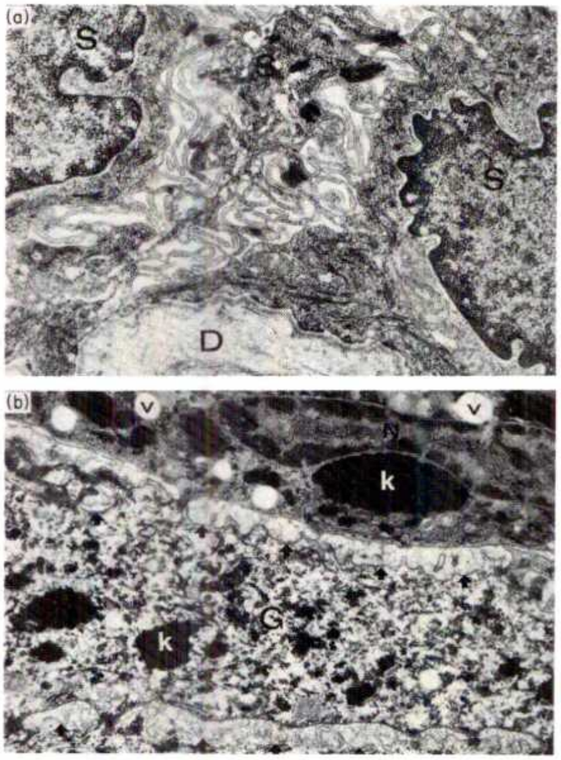

FIGURE 7. (a) 8 weeks of retinoid treatment of PRP. Microvilli in abundance with sparse desmosomes between basal and prickle cells (S). D = papillary dermis (× 13,000). (b) 4 weeks of retinoid treatment of PRP. Keratinosome-derived lamellae in abundance (arrow) in the intercellular space. G = upper stratum granulosum cell with keratohyalin (k); N = nucleus of parakeratotic cell; v = lipid-like vacuole (× 10,800).

the end of therapy. Langerhans cells were more often encountered than in untreated PRP and showed more signs of cellular activity. The papillary dermis remained mainly unchanged with slightly enlarged vessels and a moderate pericapillary infiltrate.

The effect of PUVA treatment

PUVA treatment resulted in activation of melanocytes and reactivation of keratinocytes parallel to the clinical deterioration of the skin in patient 5.

DISCUSSION

The role of vitamin A deficiency in the aetiology of PRP has been the subject of considerable study, but the relationship between PRP and vitamin A deficiency remains unproven (Leitner & Ford, 1947). Recently Finzi *et al.* (1981) reported that the level of retinol-binding protein (RBP) was markedly reduced in every PRP patient examined (eleven patients) and in some of their relatives. They postulated that defective synthesis of retinol-binding protein would be a biochemical marker for PRP. The normal RBP values in our patients show that reduced RBP is not a specific marker for all PRP patients.

Synthetic retinoids (Peck *et al.*, 1978; Lauharanta & Lassus, 1980; Fleissner & Happle, 1981;

Marks *et al.*, 1981; Pochi, 1982) and toxic doses of vitamin A (Randle, Diaz-Perez & Winkelmann, 1980) have been shown to improve PRP. The histological improvement is less conspicuous than the clinical, as it is after etretinate therapy of psoriasis (Kanerva *et al.*, 1982c) and lamellar ichthyosis (Kanerva *et al.*, 1983). The effect of PUVA therapy in PRP is controversial. Brenner *et al.* (1978) reported a beneficial effect of PUVA treatment in PRP, but in our patients PUVA caused an exacerbation. Since spontaneous remission can occur in PRP, it is difficult to evaluate the true therapeutic value of etretinate in PRP, but since other treatments, including methotrexate (Griffiths, 1980), have been disappointing, retinoids seem to be a good choice in the treatment of recalcitrant PRP.

Retinoids modulate the growth and differentiation of epithelial tissues (Elias & Williams, 1981; Orfanos *et al.*, 1981; Voorhees & Orfanos, 1981; Lauharanta, 1982), but the mechanism is unknown. In the present study we noted an increase of intercellular substance, keratinosomes and Langerhans cells and a decrease of hyperkeratosis.

We have previously found an increase of intercellular substance in the epidermis after retinoid treatment in psoriasis (Kanerva *et al.*, 1981a) and Darier's disease (Lauharanta, Niemi & Lassus, 1981). It has been suggested that retinoids might switch off keratin genes and turn on genes that specify synthesis of mucus-like intercellular material (Voorhees & Orfanos, 1981). In addition to this possible influence at the gene level retinoids can stimulate post-translational glucoconjugate synthesis at the epidermal cell surface (King & Tabiowo, 1981). However, as reported now (Fig. 4) and previously (Kanerva *et al.*, 1981, 1983), the intercellular substance sometimes appears in large amounts before retinoid treatment and might represent plasma protein exudate intermingled with cell organelles rather than any cell surface-associated material.

Keratinosomes are increased in hyperproliferative disease (Odland & Holbrook, 1981). The further increase of keratinosomes during treatment might indicate a specific action of retinoid during differentiation of keratinocytes, resulting in a decrease of corneocyte attachment and thus in a desquamative effect (Woo-Sam, 1979; Kanerva *et al.*, 1981, 1982c,d).

The stimulation of Langerhans cells (Tsambaos & Orfanos, 1981; Kanerva *et al.*, 1983) and the inhibition of lectin-induced lymphocyte mitogenesis (Bauer & Orfanos, 1981) suggests that retinoids modulate the immunocompetent cells. Langerhans cells might be involved in keratinization (Schweizer, 1981; Kanerva *et al.*, 1983) in addition to their macrophage-like function (Stingl, 1980).

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Humoral and cell-mediated immunity to human papillomavirus type 1 (HPV-1) in human warts

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SUMMARY

The humoral and cell-mediated immune response to human papillomavirus type 1 (HPV-1) has been studied in 162 patients carrying papillomas of various clinical types: deep plantar wart or myrmecia, common wart, flat wart, and anogenital wart. Circulating antibodies were detected by immunodiffusion and microcomplement fixation, using purified HPV-1 particles as type-specific antigen. A significant association between myrmecia and anti-HPV-1 antibodies was found (39% of the cases).

Cell-mediated immunity was evaluated by a study of delayed hypersensitivity (DH). The main capsid components of HPV-1 (HPV-1 CP), consisting mostly of a polypeptide of molecular weight 54,000, were injected intradermally. In addition to the type-specific antigens, HPV-1 CP contain other antigenic determinants shared by various types of human papillomaviruses and masked in intact viral particles. The DH tests to HPV-1 CP showed no differences between the carriers of different papilloma types, confirming the presence of common antigenic determinants. Moreover, they gave rise to an increase or to new anti-HPV-1 antibody production mostly in myrmecia carriers (78% and 33% of the cases, respectively), and to new DH to HPV-1 CP in all groups of papilloma carriers (33% to 56%, depending on the clinical papilloma type).

Recent biochemical and serological studies have shown the existence of at least eight types of human papillomaviruses (HPV) which differ genetically and antigenically (Gissmann, Pfister & Zur Hausen, 1977; Orth, Favre & Croissant, 1977; Orth *et al.*, 1978a,b, 1980; Gissmann & Zur Hausen, 1980; Pfister *et al.*, 1981). The available data suggest a relation between the

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morphology of the papillomas and the type of virus involved (Orth *et al.*, 1978a,b, 1980, 1981; Kienzler *et al.*, 1979; Kienzler, 1980; Kienzler, Orth & Laurent, 1981; Laurent *et al.*, 1982; Jablonska *et al.*, 1980).

This suggests that the studies so far performed on the immune response to infection by these viruses should be re-evaluated. Indeed, with the exception of recent studies in which highly purified particles of well-defined virus type were used (Pfister & Zur Hausen, 1978; Orth *et al.*, 1980; Jablonska *et al.*, 1980), most authors used as antigens viruses obtained from mixtures of lesions differing in location or clinical type, and/or lesions from different patients; thus they were heterogeneous antigens (Ogilvie, 1970; Genner, 1971; Cubie, 1972; Pass & Maizel, 1973; Morison, 1975; Pyrhönen & Johansson, 1975; Shirodaria & Matthews, 1975; Lee & Eisinger, 1976; Viac *et al.*, 1977a; Viac, Thivolet & Chardonnet, 1977b). In addition, in most cases the clinical characteristics of the papillomas studied were insufficiently described.

We now report the results of a study on the immune response of papilloma carriers to HPV type 1 (HPV-1). We have assessed the clinical type of the lesions, and used a highly purified preparation of HPV-1 as antigen for the humoral immunity (HI) studies. Cell-mediated immunity (CMI) studies were performed using as antigen the capsid polypeptides of HPV-1 (HVP-1 CP), mainly a polypeptide with a molecular weight (MW) of 54,000 (Favre *et al.*, 1977; Orth *et al.*, 1978c).

METHODS

Patients

The study included 162 patients (seventy-seven women and eighty-five men) with ages ranging from 6 to 72 years. The number of warts per patient varied from 1 to 40, and the length of the clinical evolution was from 10 days to 8 years. Application of the clinical criteria previously discussed (Agache & Laurent, 1976; Kienzler, 1980; Kienzler *et al.*, 1981) gave the following classification: deep plantar warts or myrmecia (44), common warts (including peri-ungual and filiform warts) without associated myrmecia (83), flat warts exclusively (15), and anogenital warts without associated myrmecia (20).

Some lesions were removed by surgery and submitted to histological examination, as well as to immunofluorescence (IF) tests in order to determine their HPV type, using guinea-pig antisera against HPV-1, HPV-2, and HPV-3, under the conditions previously described (Orth *et al.*, 1977, 1978b; Orth, Breitburd & Favre, 1978c).

The control groups included 110 blood donor soldiers, aged 18–20, for the HI studies, and fifty subjects, aged 20–35, in good health and with no known wart history for the CMI studies.

Antigens

A highly purified suspension of HPV-1 particles (1·35 mg/ml), obtained from a pool of myrmecia, was prepared as previously described (Favre *et al.*, 1975a) and used for the HI studies. The viral DNA, extracted from an aliquot of this preparation and analysed with restriction enzymes (Favre *et al.*, 1975b), indicated that at least 99% of the preparation consisted of HPV-1 particles.

A solution of HPV-1 capsid polypeptides (HPV-1 CP) in 0·2 M sodium carbonate buffer pH 9, was prepared as previously described by alkaline disruption of HPV-1 particles (Favre *et al.*, 1977) and used for CMI studies. The preparation contained mostly the major capsid component (MW 54,000) and a minor component (MW 76,000), as shown by electrophoresis in SDS polyacrylamide slab gel (Favre *et al.*, 1977; Orth *et al.*, 1978c).

A suspension of 20×10^6/ml β-haemolytic streptococci (Laboratoire des Stallergènes, France), as well as solutions of 1/10,000 candidin (Institut Pasteur Production, France), 20 units/ml PPD tuberculin (Statens Serum Institut, Denmark), and 10 μg/ml phyto-haemagglutinin A (PHA, Wellcome Laboratory, Great Britain), were used for CMI studies against other antigens.

Sera

Blood samples were taken at the time of the delayed hypersensitivity (DH) test, and/or of curettage (myrmecia), cryotherapy or topical use of podophyllin (other papillomas), as well as 21 days later. The sera were stored at −60°C. A hyperimmune anti-HPV-1 antiserum (G 200) (Orth *et al.*, 1978c) was used as positive control in immunodiffusion (ID) assays (titre:1/1280) and in complement fixation (CF) tests (titre:1/5120).

Humoral immunity

ID tests were performed as previously described (Orth *et al.*, 1977) using a suspension of HPV-1 at the optimal concentration of 0·7 mg/ml. CF tests were performed according to the method of Cikes (1975), the haemolytic activity of the complement being evaluated visually (Hefton & Eisinger, 1977). The optimal concentrations of complement (1 U/25 μl) and of antigen (4 U, or 0·1 μg/25 μl) were determined by cross titration (Cikes, 1975). The presence of anti-complement antibodies may induce false-positive sera and make this method unreliable for low antibody titres. Therefore only sera with a titre equal to or greater than 1/10 were considered as positive.

Cell-mediated immunity

The CMI was evaluated by DH tests. Most patients were given intradermal injections in their back of 0·05 ml of the various antigen solutions. The solution of HPV-1 CP was used at a concentration of 0·1 mg/ml, but twenty patients were given also concentrations 10- and 100-fold lower. The injection sites were examined 48 to 72 h later. The reactions were considered positive when erythema and a papule of at least 3 mm in diameter were present, or when a papule with a diameter of at least 3 mm greater than that due to carbonate buffer alone was present (in three cases). The DH tests to other antigens were considered as positive when erythema and papule had a diameter greater than 5 mm. Some patients received a second injection of HPV-1 CP after 21 days.

The χ^2 test was used for statistical analysis. The differences between two groups were considered as significant when the χ^2 value gave a probability $P < 0{\cdot}05$.

RESULTS

Humoral immunity. The frequency of antibodies against HPV-1 in the sera of carriers of various papilloma types is shown in Table 1. There is good agreement between the ID and the CF results. A significantly higher number of myrmecia carriers showed antibodies than did those with common warts ($P < 0{\cdot}001$), flat warts ($P < 0{\cdot}05$), anogenital warts ($P < 0{\cdot}05$), or control subjects ($P < 0{\cdot}001$). No significant differences were found between carriers of common, flat, or anogenital warts, or control subjects.

No significant differences were observed between the average titres of the different groups, i.e. the average of the titres expressed as the reciprocal of the highest dilution of the sera giving a precipitin line (Orth *et al.*, 1977) or preventing 80% of the haemolysis (Cikes, 1975).

TABLE 1. Frequency of anti-HPV-1 antibodies determined by immunodiffusion (ID) and complement fixation (CF) in relation to the clinical type of warts

	Frequency of anti-HPV-1 antibodies*				
	Myrmecia (n = 44)	Common warts (n = 83)	Flat warts (n = 15)	Anogenital warts (n = 20)	Control subjects (n = 110)
ID-positive sera	36	10	7	10	10
CF-positive sera	34	7	7	5	9
ID- or CF-positive sera	39	11	7	10	11

* The frequency is expressed as the percentage of the total number of patients studied (n).

Determined by ID and CF these were, respectively: 6·5 and 52 (range 1–32 and 20–160) for myrmecia carriers; 11·1 and 30 (range 1–32 and 10–40) for common wart carriers; 2·5 and 16·7 (range 1–8 and 10–40) for controls.

The highest frequency of patients showing antibodies was found in the group of 11- to 20-year-olds. No correlation was observed between the presence of antibodies and the number of lesions or the duration of disease.

Cell-mediated immunity. The results of DH to HPV-1 CP studies in papilloma carriers are shown in Table 2. The frequency of a DH reaction after injection of 5 μg of HPV-1 CP was significantly higher in myrmecia carriers than in control subjects (P < 0·05), or flat wart carriers (P < 0·02); however, there were no significant differences between myrmecia, common wart carriers, and anogenital wart carriers, or between flat wart carriers and controls. The first twenty patients studied were tested with doses of 5, 0·5, and 0·05 μg of antigen. While five patients reacted to 5 μg, only four patients reacted to 0·5 μg, and none to 0·05 μg. An important fraction of the subjects who looked anergic during the first examination developed a DH reaction after a second test, independently of the clinical type of their lesions.

Hypersensitivity to the antigens usually used in CMI studies was observed with a similar frequency in the various groups, including the control group.

Evolution of the anti-HPV-1 antibodies after intradermal injection of HPV-1 capsid polypeptides (HPV-1 CP). The initial frequencies of anti-HPV-1 antibodies in the subjects studied at the time and after the DH test to HPV-1 CP (Table 3) were very close to those noticed in all the wart carriers investigated (Table 1).

Increased antibody titres were observed only in myrmecia carriers (75% of the cases). The average titres of the sera, as determined by ID and CF, increased from 9·1 to 44 (range 1–32 and 8–128) and from 46·7 to 177 (range 20–80 and 10–640), respectively.

The proportion of sera becoming positive after the DH test was not statistically different between myrmecia and common wart carriers. Their average titres, as determined by ID and CF, were 16·7 and 45·5 (range 1–32 and 10–80) for myrmecia carriers, 3·2 and 26·7 (range 1–4 and 20–40) for common wart carriers.

TABLE 2. Frequency of delayed hypersensitivity (DH) skin reaction to HPV-1 capsid polypeptides (CP) in patients with different types of warts

	Frequency of positive DH test				
	Myrmecia	Common warts	Flat warts	Anogenital warts	Control subjects
First CP DH test	40* (30)	39 (75)	7 (15)†	25 (20)	14 (50)
Second CP DH test‡	33 (12)	33 (18)	33 (6)	56 (9)	ND
Ubiquitous antigens DH tests§	80 (30)	87 (35)	66 (15)	80 (20)	86 (50)

* The frequency is expressed as the percentage of the total number of patients studied, in parentheses.

† Two patients were immunosuppressed (renal transplant, lymphosarcoma).

‡ The second DH test is shown only for subjects found negative in the first test.

§ Patients with a positive DH test to at least one of the antigens: candidin, β haemolytic streptococcus, PPD tuberculin, PHA, were scored as positive.

ND = not determined.

TABLE 3. Variations in the titres of HPV-1 antibodies after DH test to HPV-1 capsid polypeptides (CP) in patients with different types of warts assayed by immunodiffusion (ID) and complement fixation (CF)

	Frequency of sera					
	Positive at the time of the DH test		With higher titre after the DH test†		Positive after the DH test	
Patients* with	ID	CF	ID	CF	ID	CF
Myrmecia	38‡ (21)	43 (21)	75 (8)	78 (9)	31 (13)	33 (12)
Common warts	6 (32)	6 (32)	0 (2)	0 (2)	13 (30)	10 (30)
Flat warts	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
Anogenital warts	0 (9)	0 (9)	0 (9)	0 (9)	0 (9)	0 (9)

* Fraction of the patients studied in Table 2, from whom two blood samples were collected, at the time of the DH test and 21 days later.

† Patients whose antibody titre variation was at least fourfold.

‡ The frequency is expressed as the percentage of the total number of patients studied, in parentheses.

Correlation between the type of virus found in the lesions and the immune response. In nineteen patients, the virus found in the lesions was identified by IF using anti-HPV-1, -2, and -3 immune sera (Table 4). The results confirm the preferential association of HPV-1 with myrmecia, HPV-2 with common warts, HPV-3 with flat warts (Orth *et al.*, 1978a,b, 1981; Kienzler, 1980; Kienzler *et al.*, 1981; Laurent *et al.*, 1982; Jablonska *et al.*, 1980) as well as the specificity of the humoral response, and the less specific character of the CMI.

TABLE 4. Correlations between the clinical aspect, the type of virus present in the lesions (as determined by immunofluorescence) and the immune response

Clinical aspect	Number of cases	Type of HPV detected			Number of cases presenting	
		1	2	3	anti-HPV-1 antibodies	a delayed hypersensitivity to HPV-1 CP
Myrmecia	5	4*	—	—	3	3
		—	1†	—	1	ND
Common wart	9	—	8	—	0	5
Flat wart	5	—	—	5	0	0

* One of the patients had also HPV-3-induced flat warts and showed neither anti-HPV-1 antibodies nor DH to HPV-1 capsid polypeptide (CP).

† This wart had the clinical aspect of myrmecia and the histological characteristics of common wart, according to the criteria previously defined (Agache & Laurent, 1976; Kienzler, 1980; Kienzler *et al.*, 1981).

ND = not determined.

DISCUSSION

It is known that there are several antigenically different types of HPV (Gissman *et al.*, 1977; Orth *et al.*, 1977, 1978a,b, 1980; Gissman & Zur Hausen, 1980; Pfister *et al.*, 1981) and that clinically different types of papillomas are preferentially associated with distinct types of papillomavirus, for example HPV-1 with myrmecia and HPV-2 with common warts (Orth *et al.*, 1978a,b, 1980, 1981; Kienzler *et al.*, 1979, 1981; Kienzler, 1980; Laurent *et al.*, 1982; Jablonska *et al.*, 1980). Consequently it seems reasonable that immunological studies should be performed with well-defined viruses on groups of patients with clinically well-characterized lesions, in contrast to most of the studies so far presented (Ogilvie, 1970; Genner, 1971; Cubie, 1972; Pass & Maizel, 1973; Morison, 1975; Pyrhönen & Johansson, 1975; Shirodaria & Matthews, 1975; Lee & Eisinger, 1976; Viac *et al.*, 1977a,b). In the present work we considered the clinical types of the lesions (Agache & Laurent, 1976; Kienzler, 1980; Kienzler *et al.*, 1981) and used as antigens for HI studies a highly purified preparation of HPV-1 (Favre *et al.*, 1975a,b), and for CMI studies the main capsid polypeptides of HPV-1 (Favre *et al.*, 1977; Orth *et al.*, 1978c). For these reasons, it is not possible to compare our results with those of most previous studies.

In a group of myrmecia carriers, patients showing no sign of regression and who had come for treatment, the presence of anti-HPV-1 antibodies was detected in 40% of the cases using two different serological techniques. These results are rather similar to those reported by Pfister & Zur Hausen (1978) for a group of papilloma carriers which included plantar and common warts (50% of the 10- to 20-year-old subjects showed anti-HPV-1 antibodies), and to those of Jablonska *et al.* (1980), for butchers with hand warts (40% of the butchers showed anti-HPV-1 antibodies). However, we have observed a low frequency of anti-HPV-1 antibodies in patients carrying other papillomas and in control subjects (10%), in contrast with the frequencies reported by Pfister & Zur Hausen (1978) in anogenital wart carriers (50%), in unselected patients (25–50%, depending on the age group), and in carcinoma carriers (38–53%). This difference could be due either to a greater sensitivity of the technique used by Pfister & Zur Hausen (1978), or to local epidemiological difference in HPV-1 infection.

As for CMI in relation to HPV, a previous study has shown a DH reaction to the injection of viral particles, extracted from a pool of plantar warts of undefined clinical type and inactivated with formalin, in 28–64% papilloma carriers, varying with the site of lesion (Viac *et al.*, 1977a). In the present study we have used as antigen capsid polypeptides obtained by alkaline disruption of HPV-1 particles and separation from DNA-histone complexes by gel filtration (Favre *et al.*, 1977), thus allowing their safe injection. These polypeptides carry type-specific antigens and also, in contrast with the viral particles, antigens common to various HPVs and masked in intact particles (Orth *et al.*, 1978c; Jenson *et al.*, 1980).

Our results confirm the existence of CMI to an HPV-1 capsid antigen in myrmecia carriers, but this CMI is less specific than HI since there are no significant differences compared with that of common and anogenital warts carriers. In addition, an intradermal injection of HPV-1 CP sensitizes an important fraction of the subjects, regardless of the clinical type of the papillomas. The lesser specificity of the CMI can be explained by reactions against common antigenic determinants of the capsid.

The booster effect observed in myrmecia carriers after injection of HPV-1 CP is most likely due to the type-specific antigenic determinant(s) of this antigen (Orth *et al.*, 1978c). The unusually high frequency of serological and skin test conversion following injection of viral antigen is in agreement with the results of Viac *et al.* (1977a,b). It confirms the high immunogenic capacity of our capsid polypeptides preparation as observed in the guinea-pig (Orth *et al.*, 1978c).

The results of the studies on the humoral response to infection by HPV-1 in subjects carrying different types of papillomas, and on the intradermal injection of HPV-1 capsid polypeptides both indicate that, at least in France, myrmecia are preferentially associated with HPV-1. In addition, these results underline that studies on immune response in human infections by papillomaviruses require well-defined antigens and clinically well-characterized lesions.

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Increased gliadin antibodies in dermatitis herpetiformis and pemphigoid

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SUMMARY

The mean titres of serum IgG and IgA gliadin antibodies were significantly increased in thirty-four patients with bullous pemphigoid, and in twenty-three patients with dermatitis herpetiformis, compared with twenty-four healthy controls. The patients with pemphigoid also had increased IgG and IgA gliadin antibodies compared with nine patients with pemphigus. The reason for the high titres of gliadin antibodies in pemphigoid is obscure. These patients may have increased intestinal permeability (suggested by the presence of β-lactoglobulin antibodies in some patients). Alternatively gliadin may somehow precipitate the autoimmune process.

Two of the commoner chronic bullous diseases, pemphigoid and dermatitis herpetiformis (DH), are characterized by the formation of subepidermal blisters. However, it has seemed unlikely that the two share a common aetiology as bullous pemphigoid is thought to be due to auto-antibodies being formed to the basement membrane of the epidermis (Jordan *et al.*, 1967), whereas dermatitis herpetiformis is known to be associated with gluten-sensitive enteropathy (Shuster, Watson & Marks, 1968).

Patients with dermatitis herpetiformis (DH) have high titres of gliadin antibodies (Kumar *et al.*, 1976; Menzel, Pehamburger & Holubar, 1978). We have reviewed patients sequentially whilst they were taking a gluten-free diet to assess the effect of the diet on the antibody titres, and this study will be reported elsewhere. In so doing, we used patients with bullous pemphigoid as controls. Bullous pemphigoid is of interest because it tends to be a self-limiting disease, and most patients are able to cease their steroid therapy within 2 years. In addition, the auto-antibody titres in the serum usually diminish so that these are unmeasurable after that time (Ahmed, Maize & Provost, 1977). The precipitating factors for pemphigoid are unknown.

We performed studies of gliadin antibodies in patients with various bullous diseases and found that not only did patients with DH have high titres of gliadin antibodies in their serum, but so also did the patients with pemphigoid; this was not so for the healthy control group nor for patients with pemphigus.

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METHODS

Patients

Thirty-four patients (nineteen female and fifteen male) with bullous pemphigoid were studied. Their ages ranged from 48–96 years. They were compared with twenty-three patients (eleven female and twelve male) with DH whose ages ranged from 20–70 years (none receiving a gluten-free diet), nine patients with pemphigus vulgaris or foliaceus (five female and four male) whose ages ranged from 38–80, and twenty-three healthy controls. The healthy controls were divided into two groups, thirteen 'young' and ten 'old' controls, the age range of the former being 22–65, and the latter 65–81; none had any evidence of either skin or gastro-intestinal disease. The main criteria for the diagnosis of the bullous diseases were as follows.

Bullous pemphigoid. (i) History and clinical examination suggestive of pemphigoid. (ii) Subepidermal blister on histological examination. (iii) On direct immunofluorescence examination, binding of IgG antibody to the basement membrane of the patient's epidermis and/or (on indirect immunofluorescence examination) the presence of IgG antibody in the patient's serum to the basement membrane of monkey oesophagus epithelium.

Dermatitis herpetiformis. (i) History and clinical examination suggestive of DH. (ii) Subepidermal blister on histological examination of the patient's skin. (iii) Demonstration of granular IgA deposits in the subepidermal region of the patient's skin (Cormane, 1967).

Pemphigus vulgaris or foliaceus. (i) History and clinical examination suggestive of pemphigus. (ii) Intraepidermal blister on histological examination. (iii) On direct immunofluorescence examination, binding of IgG antibody to the membrane of the keratinocytes in the epidermis of the patient's skin and/or (on indirect immunofluorescence examination) the presence of IgG antibody in the patient's serum to the membrane of the epithelial cells of monkey oesophagus.

Assays for gliadin and β-lactoglobulin antibodies

These were carried out using an enzyme-linked immunosorbent assay (ELISA) for the measurement of IgG and IgA antibodies against gliadin, and IgG antibodies against β-lactoglobulin.

The antigens used were (i) crude gliadin obtained from Dalgety Spillers, Cambridge, U.K. This was the same antigen as that used in the studies of Kieffer *et al.* (1981) and was extracted from wet-butanol-defatted strong bread-making flour using 70% ethyl alcohol. The gliadin was dissolved and diluted (50 μg/ml) in 70% ethyl alcohol (ii) β-lactoglobulin obtained from the National Institute for Research in Dairying, Reading, U.K., 10 μg/ml in carbonate buffer 0·5 M pH 9·6.

The enzyme-conjugated antibodies were obtained from Dako Laboratories, Copenhagen, Denmark. Peroxidase-coupled rabbit immunoglobulins specific for human γFc, αFc, μFc and δFc were used in the assays.

The ELISA was carried out as follows. The wells of plastic micro-ELISA plates were coated with antigen by adding 75 μl of the antigen solution (50 μg/ml) to each well. The plates were left at room temperature overnight and then washed three times in phosphate-buffered saline pH 7·3, with Tween 20 0·08% w/v (PBS-Tween 20). Serial doubling dilutions of sera in PBS-Tween 20 were added and incubated for 2 h at 4° C. Then, after the plates had been washed three times in PBS-Tween 20, the peroxidase-coupled anti-human immunoglobulins were added (75 μl per well) and incubated for 2 h at 4°C. After 6 washes, the colour reagent (ortho-phenylenediamine in citric acid buffer pH 5·0) was added (75 μl per well) and left on the plate for 30 min at room temperature. The colour reaction was stopped by the addition of 3 M H_2SO_4, and the intensity was then read on a 'multiscan through-plate reader' at 492 nm. Controls included serum added to antigen-free wells and antigen-coated wells not treated with serum. The absorbance readings were plotted against serum dilutions. Using a standard positive serum, a titration curve was constructed and the end-point taken as the serum dilution having an absorbance falling in the mid-range of the linear part of the curve. The absorbance given by this 'standard dilution' was then measured in all subsequent tests, and used as the 'cut-off' value for end-point determinations. The absorbance of the standard dilution was found to vary between $\pm 1 \log_2$ dilution, and thus was within the limits of the experimental error of the doubling dilution procedure.

Assays for antibodies to cytomegalovirus, measles virus and adenovirus

The assay for cytomegalovirus was carried out by an indirect ELISA and those for measles virus and adenovirus were carried out using a complement fixation text. These assays were carried out by Dr E.Edmond, Department of Bacteriology, University of Edinburgh.

Statistical analysis. The statistical significance of the differences in titres of antibodies to gliadin and to lactoglobulin between groups was calculated using the Mann–Whitney U test.

RESULTS

Gliadin antibodies

IgG gliadin antibodies. The titres of IgG gliadin antibodies in both the pemphigoid and DH groups were significantly higher than those in the healthy control groups and the pemphigus group (Fig. 1). (DH and pemphigoid groups compared with controls: $P < 0{\cdot}001$ for both. DH and pemphigoid groups compared with pemphigus group: $P < 0{\cdot}01$ for both).

IgA gliadin antibodies. The titres of IgA gliadin antibodies in both the pemphigoid and DH groups were significantly higher than those in the healthy control group (Fig. 2). In only the pemphigoid patients were the titres significantly higher than the pemphigus group. (DH and pemphigoid groups compared with controls: $P < 0{\cdot}001$ for both. Pemphigoid group compared with pemphigus: $P < 0{\cdot}05$).

IgM and IgD gliadin antibodies. The titres of these antibodies did not differ in any of the bullous disease groups from the control group.

β-lactoglobulin antibodies

IgG β-lactoglobulin antibodies. The titres of these antibodies were significantly higher in the DH group than in the control group ($P < 0{\cdot}02$). The pemphigoid group did not have significantly raised titres when compared with the controls, but eight of the twenty-eight patients tested had high titres (two of these very high). Neither DH nor pemphigoid groups had significantly higher titres than the pemphigus group (Fig. 3).

Antibodies to cytomegalovirus, measles virus and adenovirus. When the sera of fifteen patients with pemphigoid were compared with twenty age- and sex-matched controls, no difference was found in the titres of antibody to each of the three viruses between the two groups.

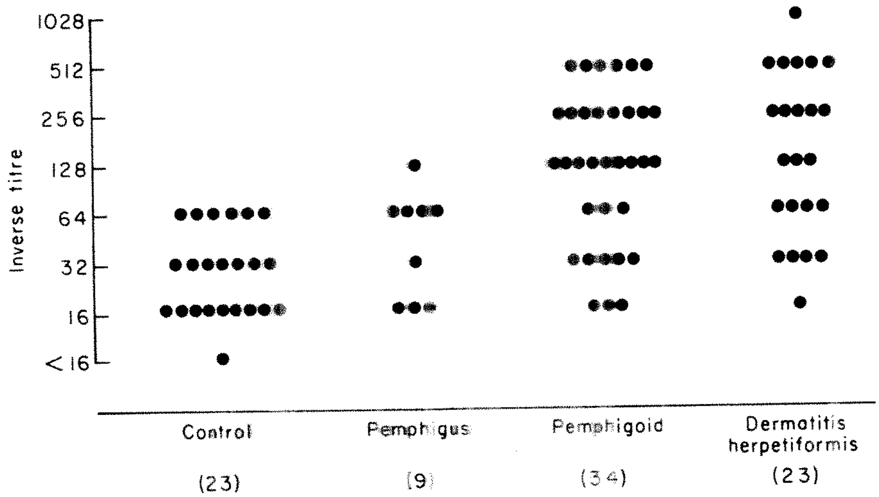

FIGURE 1. IgG gliadin antibody titres in patients with dermatitis herpetiformis, bullous pemphigoid, pemphigus and healthy controls.

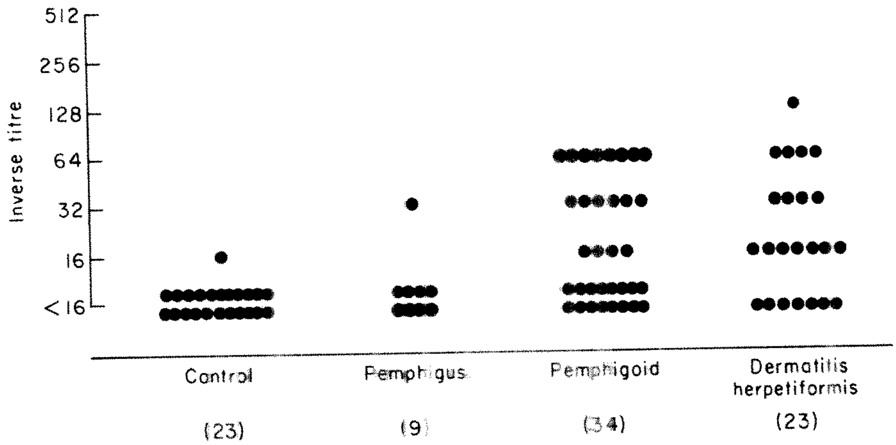

FIGURE 2. IgA gliadin antibody titres in patients with dermatitis herpetiformis, bullous pemphigoid, pemphigus and healthy controls.

DISCUSSION

These studies have shown that patients with bullous pemphigoid and dermatitis herpetiformis have high titres of gliadin antibodies in their serum. It has been reported previously that patients with gluten-sensitive enteropathy, whether due to coeliac disease or dermatitis herpetiformis (Kumar *et al.*, 1976; Menzel *et al.*, 1978), have raised titres of both IgG and IgA gliadin antibodies. It has been shown that patients with Crohn's disease and ulcerative colitis, where the intestinal mucosa might be assumed to be unduly permeable also have high titres of gliadin antibodies, though these were restricted to IgG antibodies (Kieffer *et al.*, 1981). This has led to the assumption that raised titres of IgA gliadin antibodies are specific for gluten-sensitive enteropathy (Unsworth *et al.*, 1981).

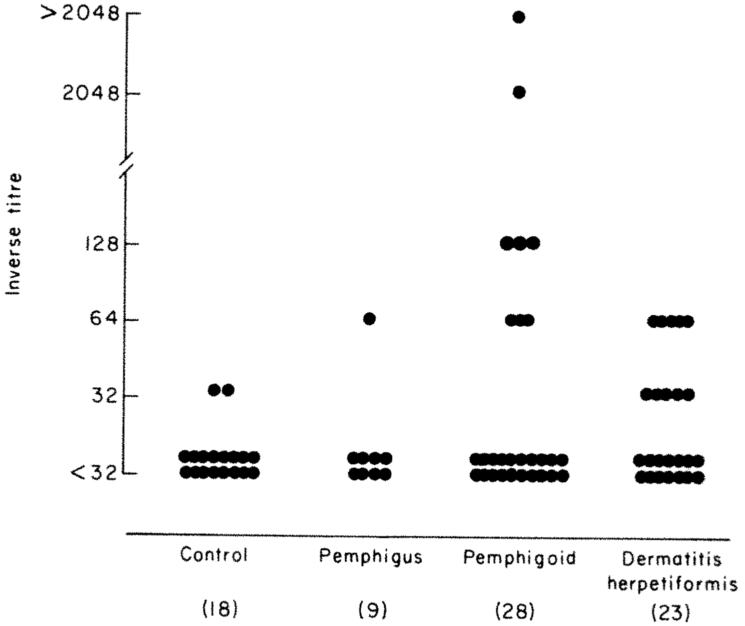

FIGURE 3. IgG β-lactoglobulin antibodies in patients with dermatitis herpetiformis, bullous pemphigoid, pemphigus and healthy controls.

The finding of raised titres of IgG and IgA gliadin antibodies in bullous pemphigoid was unexpected. In this disease there is no evidence of intestinal pathology; patients do not have intestinal symptoms such as abdominal pain or diarrhoea, and Marks & Shuster (1969) have demonstrated that jejunal biopsies are normal. This does not however rule out the possibility that there might be increased permeability of the intestinal mucosa as has been shown to be the case in atopic eczema (Jackson *et al.*, 1981) where the jejunal mucosa is normal (Barnetson, Merrett and Ferguson, 1981). This is supported to some extent by the finding of raised titres of β-lactoglobulin antibodies in a proportion of patients with pemphigoid. Raised titres of antibodies to milk and other food allergens have previously been reported in gluten-sensitive enteropathy, (Ferguson & Carswell, 1972) and have been assumed to be due to increased gut permeability resulting from jejunal mucosal atrophy. It will therefore be important to measure intestinal permeability directly in pemphigoid patients, and also to do further studies of other food allergens and enteric allergens.

Studies of antibodies to measles virus, cytomegalovirus and adenovirus were also carried out. Elevated measles antibody titres have been reported in a number of auto-immune diseases, noticeably in systemic lupus erythematosus (Phillips & Christian, 1970; Hollinger *et al.*, 1971) and in chronic active hepatitis (Triger *et al.*, 1972; Closs *et al.*, 1973). Elevated cytomegalovirus antibody titres have also been demonstrated in chronic active hepatitis and in primary biliary cirrhosis (Triger, Kurtz & Wright, 1974). No adequate explanation for the elevated titres of virus antibodies in these auto-immune diseases has been found, but it was considered worthwhile to see if viral antibodies titres were elevated in patients with pemphigoid; however, this was not the case.

Despite the fact that DH and pemphigoid are characterized by the formation of subepidermal blisters in the skin, which has led to the surmise that the diseases may be related, immunofluorescence and ultrastructural studies have shown that the skin pathology differs in the two conditions. There is good evidence that pemphigoid is an organ-specific auto-immune disease, albeit that the auto-immune process is often relatively transient and the precipitating factors for the disease have not been elucidated. The finding of raised titres of gliadin antibodies in such patients is hard to explain but it is possible that gliadin might play a part in the pathogenesis of the disease, and if this is so sequential studies might be helpful.

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Bleomycin-induced cutaneous toxicity in the rat: analysis of histopathology and ultrastructure compared with progressive systemic sclerosis (scleroderma

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SUMMARY

Rats injected with bleomycin over a 58-week period developed weight loss, alopecia, hyperpigmentation, skin thickening and skin tautness when compared with saline-injected control animals. The only significant abnormality in laboratory blood tests was an increased sedimentation rate in the bleomycin-treated rats compared with controls. Histological examination of dorsal skin showed atrophied sebaceous glands and increased collagen fibres, with diameters ranging from 37·5 to 75 nm as compared with 72·5 to 100 nm in control animals. Chronic bleomycin exposure produces clinical, histological and ultrastructural skin changes similar to those found in human progressive systemic sclerosis (scleroderma).

Bleomycin is an antitumour antibiotic (Umezawa, 1974; Ichikawa, 1970). It binds Fe (II) and facilitates oxidation of Fe (II) to Fe (III) generating free radicals in proximity to DNA with resultant DNA degradation (Lown & Joshua, 1980; Oppenheimer, Rodriguez & Hecht, 1980). Bleomycin does not have significant myelosuppressive effects, but the dosage is limited by pneumonitis progressing to pulmonary fibrosis and cutaneous fibrosis (Carter, 1978). The predilection of bleomycin toxicity for the lung and skin results in part from increased

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accumulation in these tissues and in part from decreased inactivation (Umezawa, 1974; Müller & Zahn, 1976).

Endothelial cell injury and altered metabolism resulting from bleomycin-induced peroxidation of the plasma membrane may initiate a non-specific injury response leading to pulmonary and cutaneous fibrosis (Burkhardt, Höltje & Gebbers, 1976; Adamson & Bowden, 1974; Catravas, Lazo & Gillis, 1981; Tom & Montgomery, 1980). Collagen biosynthesis and degradation in response to bleomycin has been extensively studied and correlated with structure and function in the lung (Collins *et al.*, 1981; Kelley, Newman & Evans, 1980; Clark *et al.*, 1980a). Clinical aspects of cutaneous toxicity of bleomycin include hyperpigmentation, alopecia, sclerosis, gangrene, oedema and fibrosis (Nixon *et al.*, 1981; Cohen *et al.*, 1973; Werner & Tornberg, 1976). Increased synthesis of type I collagen has been observed using *in vitro* cultures of fibroblasts with bleomycin (Clark, Starcher & Uitto, 1980b).

TABLE 1. Toxic effects of bleomycin on skin, fur and weights of bleomycin-treated rats (T) compared with control rats (C)

	Total dose		Skin texture		Pigmentation		Fur		Mean wt (g)		Weight change	
Weeks	T	C	T	C	T	C	T	C	T	C	T	C
0	0	0	Normal	Normal	–	–	Normal	Normal	277·2	277·8		
4	30 u/kg	0	Normal	Normal	–	–	Normal	Normal	280·5	299·6	+1·2%	+7·8%
10	45 u/kg	0	Taut & tough	Normal	+	–	Thin	Normal	269·2	335·2	–4%	11·8%
58	75 u/kg	0	Taut & tough	Normal	–	–	Very thin	Normal	308·5	351	+14·5%	+4·7%

Finch *et al.* (1980) reported increased collagen production by dermal fibroblasts from patients with cutaneous bleomycin toxicity and suggested bleomycin toxicity as a model for progressive systemic sclerosis (PSS). Campbell & LeRoy (1975) have proposed a vascular injury hypothesis for PSS leading to mononuclear cell infiltrate, fibroblast proliferation and collagen production. Fibroblast proliferation as a cell-mediated immune response has been proposed for bleomycin toxicity (Finch *et al.*, 1980) and PSS (Kondo, Rabin & Rodnan, 1976) but bleomycin toxicity has been observed in athymic 'nude' mice (Szapiel *et al.*, 1979). The skin thickening in PSS may be due to both increased fibroblast proliferation and collagen production (Buckingham *et al.*, 1978; Botstein, Sherer & LeRoy, 1982) and decreased collagen degradation (Brady, 1975; Jimenez, Yankowski & Frontino, 1977). Collagen fibres in PSS have a narrowed fibre diameter and an increased number of reducible cross-links compared with normal mature dermal collagen (LeRoy, 1974; Hayes & Rodnan, 1971). The ratio of type I to type III collagen production in PSS is reported to be normal *in vivo* (Lovell *et al.*, 1979) and *in vitro* (Uitto, Bauer & Eisen, 1979). Fleischmajer *et al.* (1978) reported increased amounts of narrow diameter type III collagen during the cellular phase of PSS which was replaced by 70 to 100 nm diameter type I collagen in the fibrotic phase of the disease. The present study was undertaken to examine the effects of long-term bleomycin toxicity in the rat, and to compare the collagen ultrastructure in bleomycin-treated rats with that seen in PSS.

METHODS

Eight 14-week-old female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Madison, Wisconsin. Sterile bleomycin sulphate (Blenoxane®) was kindly provided by Bristol Laboratories, Syracuse, New York 13201. It was dissolved in 0·85% sterile saline for injection.

Four rats were injected subcutaneously with bleomycin and four rats were injected with 0·85% sterile saline to serve as control animals. The animals were evaluated bi-weekly by the following criteria: condition of the fur, texture and pigmentation of the skin, and weight. The dose and frequency of the injections were adjusted according to the condition of the rats as determined by these examinations. After three injections of 10 units/kg (u/kg) bleomycin at 2-week intervals, the animals failed to show normal weight gain and were lethargic, so the dose was reduced to 5 u/kg injected at 2-week intervals. After three injections at this dosage, the animals showed a loss in weight, skin and fur changes, and dyspnoea, so the treatment was reduced to one injection of 5 u/kg every 8 weeks for six injections, resulting in a total cumulative dose of 75 u/kg. Rats maintained on this dosage gained weight but continued to show changes in skin and fur as well as dyspnoea.

Laboratory studies

After 58 weeks of treatment the rats were injected with a lethal dose of 30 mg/kg sodium pentobarbital. Before death, blood was drawn by cardiac puncture for laboratory tests. Red blood cell count, white blood cell count and differential, haematocrit and haemoglobin were measured by standard laboratory techniques. Sedimentation rates were determined by the Westergren method. Rheumatoid factors were determined by the RheumaQuik® (Biological Corporation of America) method. Fluorescent antinuclear antibodies (FANA) were determined using cultured Vero cells as substrate (Parker & Kerby, 1974). Circulating immune complexes (IC) were determined by the fluorescent Raji cell assay. Serum was frozen at −70°C, thawed once and assayed as described by Theofilopoulos *et al.* (1974). Dorsal cutaneous tissue was excised at least 3 cm from the site of the bleomycin injections. Tissue for light microscopy was fixed in Zenker's fluid, embedded in paraffin, sectioned at 10 μ, and stained with haematoxylin and eosin. Tissue for electron microscopy was fixed in 3% glutaraldehyde in 0·1 M phosphate buffer, pH 7·3; postfixed in 2% OsO_4 in 0·1 M phosphate buffer, pH 7·3; dehydrated through ethanols; and embedded in araldite or epon araldite. Thin sections were cut and stained with uranyl acetate and lead citrate. Sections from the upper dermis were examined on a Zeiss 95-2 electron microscope.

RESULTS

The rats were first evaluated before treatment with bleomycin or saline. All rats had thick, dense fur, and unpigmented skin. The initial mean weights of the control rats and the bleomycin-treated rats were 277·8 g and 277.2 g respectively. After 4 weeks of treatment during which the animals received 30 u/kg bleomycin there was no change in skin or fur but the mean weight of the bleomycin-treated rats increased by only 1·2% compared with a 7·8% increase for the control rats. After three more injections of 5 u/kg (total dose 45 u/kg over 10 weeks), the bleomycin-treated rats had lost weight and showed scleroderma-like changes in skin texture and pigmentation. The mean weight of the untreated animals was 335·2 g (an increase of 11·8%) while the mean weight of the bleomycin treated rats was 269·2 g (a 4% loss). The fur and skin of the control animals showed no change. The fur of the treated animals was slightly thinned, but more notable were areas of hyperpigmentation on the trunk and the tail (Fig. 1). The skin was taut and tough, making it difficult to pinch or inject with a twenty-one gauge needle.

After three injections, their frequency was reduced to once every 8 weeks while the dose

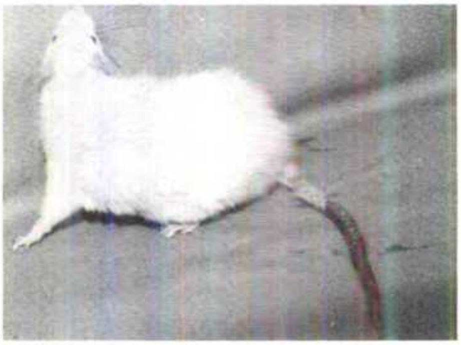
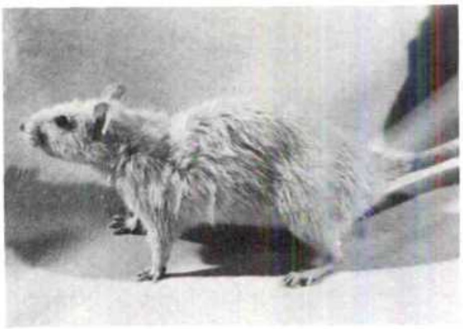


FIGURE 1. Bleomycin-treated rat after 10 weeks. Note thinning of hair and hyperpigmentation of tail.

remained at 5 u/kg. After a further six injections (total dose 75 u/kg over a 58-week period), the animals were sacrificed. The skin showed continued diffuse alopecia and tautness, but the hyperpigmentation had resolved with reduction in the frequency of bleomycin injections (Fig. 2). The untreated animals still had thick, smooth fur with no pigmentation or tautness (Fig. 3). The weight of the control rats had increased by 4·7% (to 351 g) while that of the bleomycin-treated rats had increased by 14·5% (to 308·5 g) with reduction of injection frequency.

Laboratory blood test results including CBC with differential and sedimentation rate were analysed by either *t* tests or the non-parametric Mann-Whitney U test. No significant differences were found in any category except the mean sedimentation rate of 2·5 which was increased for bleomycin-treated rats compared with the mean value of 0·5 for the control rats ($P < 0{\cdot}01$). The normal sedimentation rate for rats is 0–1. IC, FANA, and rheumatoid factors were all negative.

When examined histologically, bleomycin-treated rats had a thinner epidermis than saline-injected rats. The dermis of the treated animals showed an increase in fibroblasts and collagen as compared with the control animals (Figs. 4 and 5). The mean number of fibroblasts found in normal animals was 9·1/mm^2 with a range from 6 to 11/mm^2. Bleomycin-treated animals had a mean of 16·2 fibroblasts/mm^2 with a range from 14 to 24/mm^2. Sebaceous glands were atrophied in bleomycin-treated rats compared with control rats. Fig. 6 shows transverse



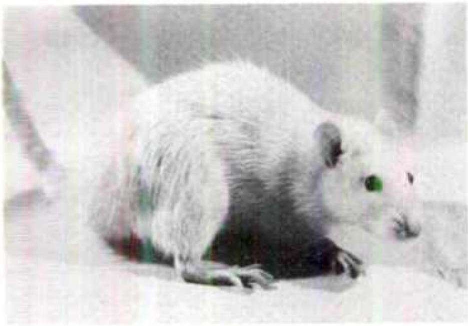
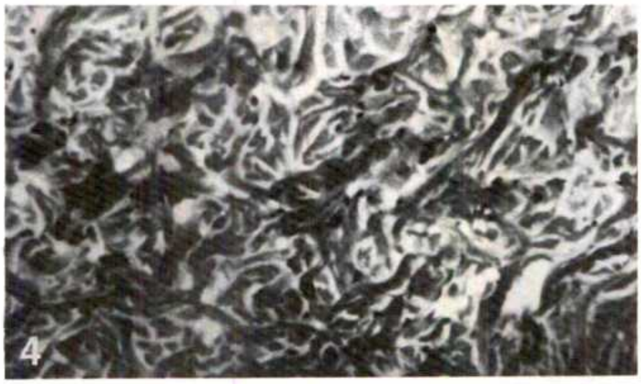


FIGURE 2. (Left) Bleomycin-treated rat after 58 weeks. Further hair and weight loss noted but hyperpigmentation no longer present.

FIGURE 3. (Right) Untreated control rat after 58 weeks.



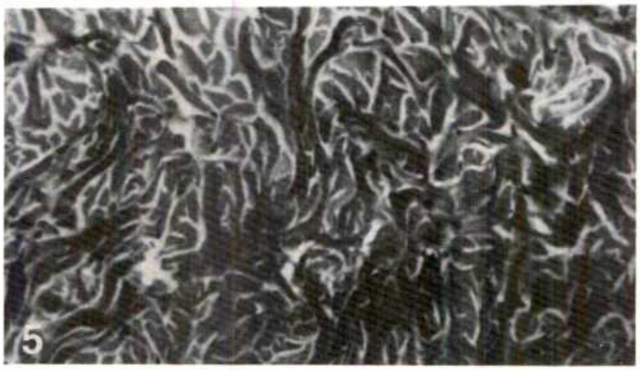


FIGURE 4. Dermis of bleomycin-treated rats shows mean fibroblast count increased to 16·2 per mm^2 in a 10 μ section.

FIGURE 5. Dermis of untreated control rat with a mean fibroblast count of 9·1 per mm^2 in a 10μ section.

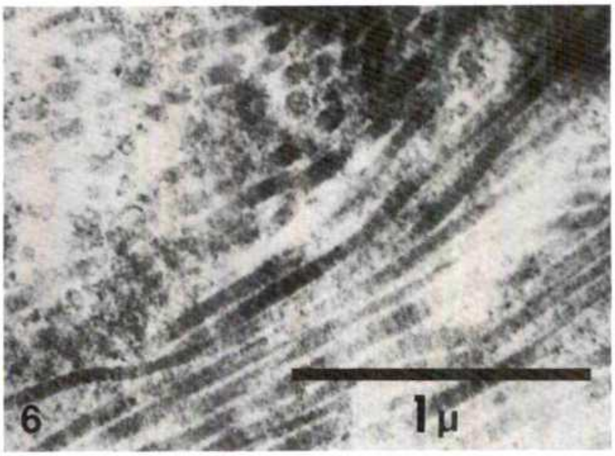


FIGURE 6. Ultrastructure of fixed collagen fibres demonstrating transverse and longitudinal view from skin of bleomycin-treated rat after 58 weeks. Note narrow fibre diameter and longer striation lengths compared with control rats (Fig. 7).

and longitudinal sections of collagen fibres viewed by electron microscopy demonstrating a 'plywood' organization (Fleischmajer & Prunieras, 1972) and an increase in thin collagen fibres which ranged from 37·5 to 75 nm in diameter. The periodicity of fibres from bleomycin-treated animals was 51·4 nm. Fibres from normal rats ranged from 72·5 to 100 nm in diameter with most fibres 100 nm in diameter. The periodicity ranged from 43·7 to 44 nm (Fig. 7).

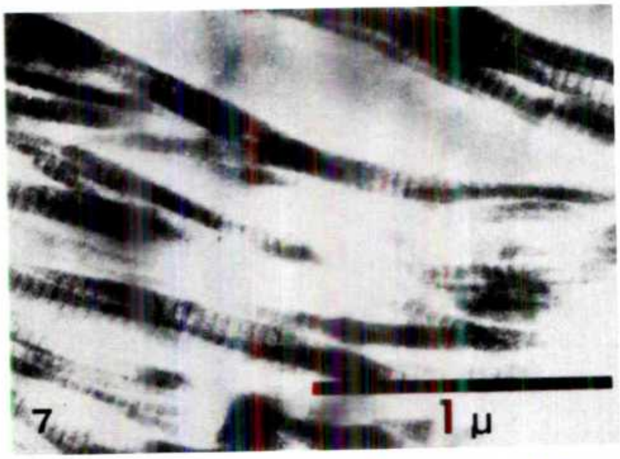


FIGURE 7. Electron microscopy of collagen fibres in control rat after 58 weeks.

DISCUSSION

We have examined the chronic cutaneous toxic effects of sublethal doses of bleomycin on rats as a model for the skin changes that occur in progressive systemic sclerosis (PSS). The rats were initially given high doses of bleomycin, but over a 1-year period, this was gradually tapered to injections of 5 u/kg every 8 weeks, and the rats were sacrificed 8 weeks after the last dose of bleomycin. The rats uniformly developed hyperpigmentation, thick taut skin, dyspnoea, weight loss, and alopecia. Hyperpigmentation and weight loss were the first changes, occurring after 45 u/kg. They were the only changes found to be reversible when the frequency of the injections was reduced. Therefore, they appeared to be dose dependent over a short time period, but not dependent on the total accumulated dose. The sclerotic changes and dyspnoea persisted in all animals after reduction in frequency of doses, suggesting fibrotic changes which are either non-reversible or reversible only after cessation of bleomycin treatment.

Under light microscopy, the skin from bleomycin-treated rats demonstrated thinning of the epidermis, loss of epidermal appendages, increased numbers of dermal fibroblasts and a homogenous deposition of collagen. The increased number of dermal fibroblasts were characterized by an extensive rough endoplasmic reticulum signifying synthesis of the procollagen helix (Leblond & Wright, 1981). There were minimal inflammatory changes and no infiltration of lymphocytes was seen in the dermis, indicating that the chronic progressive increase in fibrosis can occur in the absence of lymphocytes as suggested by previous experiments with athymic nude mice (Szapiel *et al.*, 1979). Clark *et al.* (1980b) reported an increased production of type I collagen after bleomycin exposure. Fleischmajer *et al.* (1978)

reported increased type III collagen in the cellular phase of PSS which was later replaced by type I collagen in the fibrotic phase of the disease. Thus, both our light microscopy results and previous biochemical analysis of collagen types support chronic bleomycin toxicity as a model for the fibrotic phase of PSS.

Previous studies have shown that after instillation of bleomycin in animal lungs, fibroblast proliferation appears after 6 days, and collagen synthesis markedly increases after 8 days but returns to normal after 8 weeks (Hesterberg *et al.*, 1981; Clark *et al.*, 1980a). Degradation of newly synthesized collagen also increases during the period of increased production so that the static level of collagen after 8 weeks represents a balance of low grade production and degradation.

Our study also investigated the ultrastructure of the static dermal collagen present after chronic bleomycin exposure. The collagen fibres were markedly reduced in diameter in the treated rats. Hayes & Rodnan (1971) reported a mean diameter of fibres in human scleroderma skin of 30 to 50 nm, while the mean diameter of fibres in normal skin was 120 nm. This population of thin fibres has been interpreted to represent newly synthesized collagen (Fleischmajer, Damiano & Nedwich, 1972). Our study has shown that the histological and ultrastructural analysis of skin from rats subjected to chronic bleomycin toxicity is similar to that of skin from patients with PSS, and this may represent a useful rat model for human PSS.

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Increased neutral protease and collagenase activity in recessive dystrophic epidermolysis bullosa

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SUMMARY

The enzyme activities of normal-looking skin and blister fluid from a patient with recessive dystrophic epidermolysis bullosa (RDEB) were measured. Of the hydrolytic enzymes measured, both collagenase and neutral protease activities were considerably increased in the skin and blister fluid samples compared with values found in normal control skin and in blister fluid from a patient with a burn. In addition, skin from a healthy person cultured with RDEB blister fluid showed dermal-epidermal separation. These findings suggest that collagenase and neutral protease may be involved in the formation of blisters in RDEB.

Recessive dystrophic epidermolysis bullosa (RDEB) is a rare hereditary disease of the skin and mucous membranes. It is characterized by the formation of severe blisters, which occur as a result of minor mechanical trauma, and dystrophic changes. Due to the recurrent blistering, scars, flexural contractures and mitten-like deformities of the hands and feet result. Mucosal and oesophageal involvement, accompanied by protein loss through the skin, greatly impair the nutritional status of these patients.

Although the cause of RDEB is unknown, ultra-structural studies show that there is a collagen degeneration and phagocytosis of collagen fibrils in the blister area (Pearson, 1962), suggesting that collagen breakdown may be the important factor in the blister formation. To support this hypothesis, it has been demonstrated that there is an increase in collagenase activity in the organ cultures of blistered skin (Eisen, 1969; Lazarus, 1972; Michaelson *et al.*, 1974), and an increase in tissue levels of immuno-reactive collagenase in both blistered and non-blistered skin (Bauer, Gedde-Dahl & Eisen, 1977). In addition, recent studies indicate that an increase in

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the synthesis of structurally altered collagenase in cultured skin fibroblasts is expressed as a specific genetic trait (Bauer, 1977; Bauer & Eisen, 1978).

In this study, the clinical course, histological, electron microscopical and immunological findings of RDEB are reported. The activities of several enzymes contained in the blister fluid and RDEB skin are also documented, as are studies on the effect of blister fluid on normal skin culture.

METHODS

Case report

A 13-year-old boy, admitted in acute distress due to dehydration, was diagnosed as suffering from RDEB. On admission, blisters were observed on the elbows and knees, with severe erosions over his entire body. He had two brothers. The older brother had no history of skin abnormalities, but the younger one suffered from the same disease. There was no history of skin disease in either parent or related family. Personal history revealed that the first blister developed at birth on an ankle, accompanied by erosion of the buttocks and trunk, with associated skin fragility. Blister formation and erosion occurred at sites of slight trauma, at times spontaneously, with slow healing being followed by atrophic scarring and milia formation. Loss of all fingernails and toenails occurred at age 6 years, with the vicious cycle of blister formation and scarring continuing, leading to digital fusion and mitten-like epidermal encasements of the fingers and toes (Fig. 1), and flexural contractures of the knee, elbow and wrist joints. The oral mucosa, due to moderate fragility, had small eroded areas though these had not interfered with feeding. The boy's general growth and development was only slightly retarded, his weight being 18 kg and his intelligence was normal. His deciduous teeth developed caries early and his permanent teeth were markedly dystrophic. Emergency treatment administered on admission included multiple blood and plasma transfusions, intravenous electrolytes and antibiotics, with local symptomatic care. As a result, his general condition and skin lesions showed signs of slight improvement. Death unfortunately occurred on the fiftieth day of hospitalization owing to inhalation of vomitus.

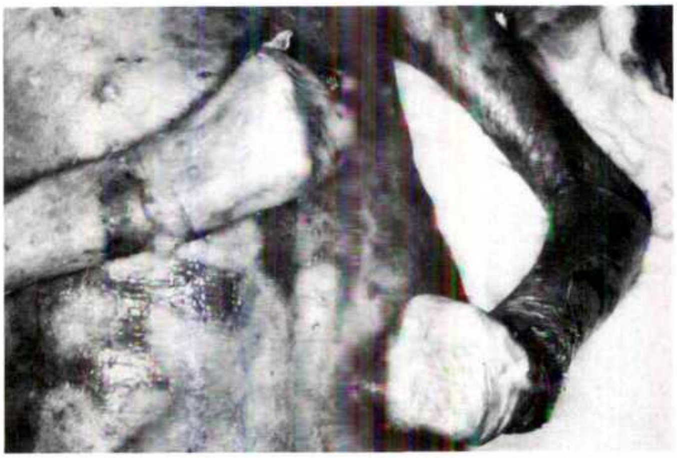

FIGURE 1. Clinical picture of a patient with recessive dystrophic epidermolysis bullosa. Hands showing contraction, syndactyly of fingers, marked scarring and nail loss.

Biopsy

A biopsy specimen was taken from non-blistered skin on the patient's abdomen and revealed subepidermal separation, without acantholysis, though inflammatory infiltrates were present in small amounts. PAS-positive basement membrane was split between the floor and roof of the subepidermal bulla. Silver positive material was shown on the roof of the blister (Fig. 2). On staining for elastic tissue, the dermal-epidermal separation and degeneration of elastic and collagen fibres in the papillary dermis were revealed. Electron microscopy of clinically non-blistered skin from a patient with RDEB showed the bulla to be localized in the superficial layers of the dermis (dermolytic blistering) and showed necrotic collagen in the blister cavity. Marked collagen degeneration could be observed in the papillary portion of the dermis, and in the same area, there was a variable loss or decrease in the number of anchoring fibrils. Vacuolar changes in the mitochondria, and other degenerative changes of the basal cells were recorded.

Immunofluorescent studies

Direct and indirect immunofluorescent studies were carried out according to the method of Beutner, Chorzelski & Jordon (1970).

Blister fluid

Blister fluid was obtained from spontaneous fresh blisters on the skin of the patient with RDEB and from a patient with burns. Blister fluid was collected every morning from a patient, and centrifuged at 3000 r.p.m. for 10 min at 4°C, to remove cell debris and/or precipitated materials. The resultant supernatants were then stored in small aliquots at −20°C until required for use either as organ cultures or for enzyme activity assay.

Preparation of skin extracts for enzyme assay

Skin samples were obtained from normal-looking areas on the patient's abdomen and from normal human abdomen obtained during surgery, and were stored at −20°C. After thawing at room temperature, the subcutaneous tissues were scraped away, the skin weighed and minced finely with scissors. The minced skin was suspended in nine parts (w/v) of 10 mM Tris-acetate

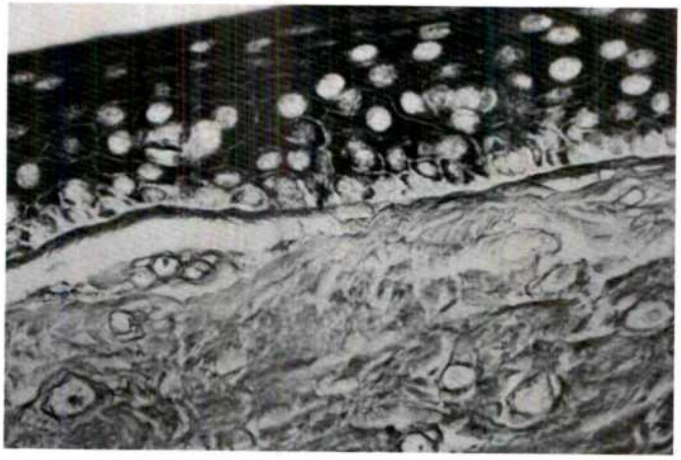

FIGURE 2. Histology of the lesional skin of a patient with recessive dystrophic epidermolysis bullosa. Silver positive material is seen on the roof of the blister. Silver stain (× 320).

(pH 7·5) with 0·85% NaCl, and the suspension homogenized with a Polytron (Brinkmann Instruments) for ten 30-second bursts, with an interval of 30 sec for cooling between each one. The homogenates were centrifuged at 18,000 × *g* for 30 min at 4°C. The supernatants were used for enzyme activity assay.

Determination of enzyme activities

Enzyme activities were measured in the blister fluids and skin extracts. Lactate dehydrogenase (LDH) was measured according to the method of Bergmeyer & Bernet (1974). Determination of acid phosphatase activity was carried out by the method of Bretz & Baggiolini (1974) using p-nitrophenol phosphate as a substrate. β-glucuronidase was measured by the procedure described by Avila & Convit (1973) using phenolphthalein glucuronidate as a substrate. Cathepsin D was assayed according to the method of Moore & Stein (1954). Elastase activity was determined by measuring the rate of p-nitrophenol liberation from N-tert-butoxycarbonyl-L-alanine p-nitrophenol ester, as described by Dewald *et al.* (1975). Neutral protease activity was determined by the procedure described by Dewald *et al.* (1975), using casein as a substrate. Collagenase activity was determined by measuring the release of soluble radioactive peptides from reconstituted [^{14}C]collagen fibrils (specific radioactivity, 16·75 d.p.m./μg). Two hundred microlitres of 0·4% [^{14}C]-labelled collagen in 0·1 M Tris:0·4 M NaCl:10 mM CaCl$_2$ (pH 7·7) and an equal volume of the sample solution were incubated at 37°C for 6 h, after which 200 μl of cold 15% TCA:1·5% tannic acid was added. The mixture was centrifuged at 3000 r.p.m. for 10 min at 4°C, and then 200 μl of supernatant solution was counted in a liquid scintillation counter. All assays were performed in triplicate, including buffer blanks. Collagenase activity was expressed as ng of collagen hydrolyzed per 1 hour.

Determination of protein concentration

The protein concentration of the blister fluids and skin extracts was measured by the method described by Lowry *et al.* (1951), with serum albumin as a standard.

Organ culture

Organ culture was carried out according to the method described by Sarkany, Grice & Caron (1965). Briefly, normal human breast or abdominal skin obtained during surgery was used. The skin was sliced to a thickness of about 0·5 mm and cut into approximately 2 × 2 mm pieces. These pieces were placed dermis side down on 20 × 20 mm paraffin-rimmed lens-paper 'rafts', which were floated in culture dishes on the top of Eagle's Minimum Essential Medium containing 60 μg/ml of kanamycin (1·0 ml final volume), and cultured in a humid atmosphere containing 5% CO$_2$ in air for 12, 24, 48 and 72 h. Previously obtained blister fluid (0·5 ml) was added to the culture medium, and after the stated times, the skin specimens were examined by routine histological methods (hematoxylin and eosin stains).

RESULTS

Immunofluorescent studies

Direct immunofluorescence, employing antisera against IgG, IgA, IgM and C3 revealed no deposition on the patient's specimens. Indirect immunofluorescence, using normal human skin as a substrate, showed no auto-antibodies in the patient's serum.

In order to examine whether the bullous pemphigoid antibody could bind to the skin from a patient with RDEB (as is the case with normal skin), the indirect immunofluorescent study was employed. Serum was obtained from a patient with bullous pemphigoid with a high titre (1:640) of anti-basement membrane antibody, and skin from the patient with RDEB was used as a substrate. The results were then compared with those of normal skin. The RDEB skin revealed immunofluorescent deposits at the basement membrane zone, as in normal skin, and it also gave the same titre of bullous pemphigoid antibody. These results suggest that the RDEB skin-binding site for the anti-basement membrane antibody is not destroyed.

Enzyme activities in skin extract

The selected enzymes were as follows: LDH as a marker enzyme for cytosol; acid phosphatase, β-glucuronidase and cathepsin D as lysosomal enzymes; and elastase, collagenase and neutral protease, all of which had an optimal pH at a neutral range. Table I shows the activities of collagenase and neutral protease, which were 2·7 and 4·9 times higher in RDEB skin than in normal skin respectively. LDH and acid phosphatase activity were approximately twice as high in RDEB skin as in normal skin. Other enzymes measured showed almost the same activities in both skins.

Enzyme activities in blister fluids

The enzyme activities in blister fluids obtained from the patient with RDEB and the one with burns are shown in Table II. There was no significant difference in the activities of LDH (the marker enzyme for cytosol) and lysosomal enzymes (acid phosphatase, β-glucuronidase, cathepsin D, and elastase) between RDEB and burn blister fluids. However, the activities of collagenase and neutral protease in RDEB blister fluid were 3·7 and 8·8 times higher than in burn blister fluid.

TABLE I. Enzyme activities of skin extract

Enzymes	Enzyme activity		Relative activity (RDEB/normal)
	Normal	RDEB	
LDH (OD_{340}/min)	1·52	2·63	1·7
Acid phosphatase (μg of p-nitrophenol/min)	4·05	8·29	2·1
β-glucuronidase (μg of phenolphthalein/h)	45·3	50·9	1·1
Cathepsin (OD_{570}/h)	6·23	7·46	1·2
Elastase ($OD_{347.5}$/min)	0·025	0·025	1·0
Neutral protease (nmoles of L-leu-leucine/h)	7·23	35·6	4·9
Collagenase (ng of collagen/h)	112·5	305·0	2·7

Enzyme activities are expressed as activity per mg protein. The normal control data is expressed as the mean of five separate preparations.

TABLE 2. Enzyme activities of blister fluid

Enzymes	Enzyme activity		Relative activity (RDEB/burn)
	Burn	RDEB	
Acid phosphatase			
(μg of p-nitrophenol/min)	0·045	0·076	1·7
β-glucuronidase			
(μg of phenolphthalein/h)	0·848	1·328	1·6
Cathepsin			
(OD_{570}/h)	0·023	0·031	1·4
Elastase			
($OD_{347\cdot5}$/min)	0·058	0·040	0·7
Neutral protease			
(nmoles of L-leu-leucine/h)	0·073	0·643	8·8
Collagenase			
(ng of collagen/h	2·95	10·88	3·7

Enzyme activities are expressed as activity per mg protein.

Effect of blister fluid on the normal skin culture

Normal human skin was cultured for 24, 48 and 72 h in the presence of blister fluids obtained from the patients with RDEB and a patient with burns. There was no remarkable histological change in the skin explant cultured with blister fluid from the burns patient. On the other hand, the skin explant cultured with blister fluid from RDEB under the same conditions, resulted in dermal-epidermal separation after culturing for 72 h, and appeared similar to the histology of RDEB *in vivo* (Fig. 3).

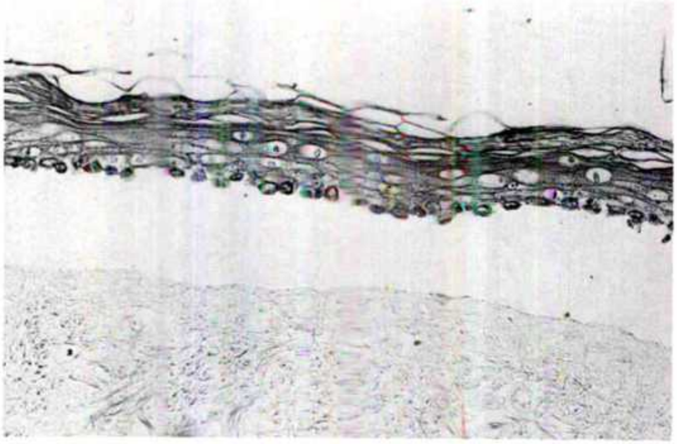

FIGURE 3. Histology of normal human skin explants cultured for 72 h with blister fluid from a patient with recessive dystrophic epidermolysis bullosa. Separation of the epidermis from the dermis is shown. Silver stain (× 160).

DISCUSSION

The pathogenesis of recessively inherited epidermolysis bullosa dystrophica remains uncertain. Eisen (1969) showed that the production of collagenase from skin cultures obtained from friction bullae in patients with RDEB was approximately six-fold greater than in normal skin. This enzyme activity was also elevated in the non-bullous skin of these patients. From these findings, he proposed that collagenase may play a direct role in the blister formation in this disease. On the other hand, Lazarus (1972) found that cultures of clinically normal skin from patients with RDEB did not differ significantly in collagenase production compared with normal healthy skin, but cultures of skin lesions produced 3 times more collagenase than normal skin from the same patient. Therefore he proposed that increased local levels of collagenase were a secondary tissue reaction to chronic injury. Recently, Eisenberg, Stevens & Schofield (1978) demonstrated a significant increase of collagenase and collagen peptidase activities in the roof of a fresh blister from RDEB skin (without culture). The collagenase activity was 2·6 to 24 times greater than normal, and collagen peptidase was 19 to 52 times greater than normal. As these figures indicate, the role of collagenase in the pathogenesis of RDEB is still unresolved. The present study was designed to assess whether any other enzyme or enzymes can be correlated with blister formation. We were able to confirm a marked increase in the activity of collagenase, and also to demonstrate the rise of neutral protease activity in the skin of RDEB patients. Blister enzyme activities gave similar results.

These findings suggest that not only collagenase but also neutral protease is responsible for the formation of blisters in RDEB skin. To confirm this hypothesis, the effect of blister fluid from the patient with RDEB on normal skin explant cultures was studied, and these showed a clean dermal-epidermal separation (Fig. 3). These results show that blister fluid from a patient with RDEB contains some specific factor(s), such as protease, which induces the dermal-epidermal separation, and they also demonstrate that light microscopical features resembling those of RDEB *in vivo* can be induced in normal skin with no congenital defects.

The above findings suggest the possibility that either collagenase and/or neutral protease may act directly or indirectly as the blister forming enzyme in RDEB.

Although the natural substrate of neutral protease is still unknown, this enzyme is capable of protein degradation under certain physical (temperature and pH) conditions, as is collagenase. The question remains as to the source of neutral protease (the upper dermis, epidermis or granulocytes). Further experiments, including electron microscopic studies using cultured skin, are now in progress.

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Urticaria and vasculitis: a continuum of histological and immunopathological changes

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SUMMARY

Histopathological criteria were used to classify twenty-four patients with chronic urticaria into three groups, which were then studied to establish whether circulating immune complexes (CICs), complement activation and deposition of immunoreactants are confined to patients with urticarial vasculitis. Group 1 (three patients) had classical urticarial vasculitis, and two of these patients showed hypocomplementaemia with evidence of C3 conversion and deposition of immunoreactants in lesional and uninvolved skin. Ten patients (group 2) with a dense perivascular mixed-cellular infiltrate had normal or raised complement levels and infrequent evidence of C3 conversion. Immunoreactants were detected only in their lesional skin. Eleven patients (group 3) had only a sparse perivascular infiltrate. In this group, complement was normal and immunofluorescence was essentially negative. Cryoglobulins were detected in group 1 patients only. Monoclonal rheumatoid factor and C1q binding were positive in all group 1 patients, half the group 2 patients and none of the group 3 patients. This study suggests that urticaria and urticarial vasculitis form a disease continuum, and identifies a group of patients with features intermediate between urticarial vasculitis and ordinary urticaria.

The term 'urticarial vasculitis' refers to patients with recurrent urticaria and the histological features of necrotising vasculitis. Several papers have described the clinical and immunopathological features (McDuffie *et al.*, 1973; Soter, Austen & Gigli, 1974; Agnello *et al.*, 1975; Agnello, Gabriel & Tai, 1976; Feig *et al.*, 1976; Gammon & Wheeler, 1979; Ludivico, Myers & Maver, 1979; McLean *et al.*, 1980; Millns *et al.*, 1980; Phanuphak *et al.*, 1980; Zeiss *et al.*, 1980; Van der Horst & Bronsveld, 1981; Callen & Kalbfleisch, 1982), but the group of sixteen patients studied by Soter (1977) is the largest series in the literature. The syndrome affects predominantly females, and it can be distinguished from ordinary urticaria since the individual lesions last longer and often fade to leave purpuric staining or other evidence of red cell extravasation. Systemic involvement is often present and is usually characterized by arthralgia, arthritis or gastro-intestinal upset. Renal disease occurs only rarely.

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Most reports have emphasized the importance of distinguishing urticarial vasculitis from systemic lupus erythematosus, which may also present with urticaria (Provost *et al.*, 1980). It has also been suggested that a significant proportion of patients with chronic idiopathic urticaria have histological features of vasculitis. Unfortunately different criteria have been used to identify cases of vasculitis. Thus Monroe *et al.* (1981) defined vasculitis on the basis of fibrinoid deposits and leukocytoclasis, whereas Phanuphak *et al.* (1980) used the presence of inflammatory cells within the walls of affected vessels. These two prospective studies produced figures of 20% and 52% for the incidence of vasculitis in chronic urticaria and they emphasize the importance of the diagnostic criteria if meaningful comparisons are to be made between studies.

We have classified our cases into three groups according to histopathological criteria, and the immunopathological findings associated with each group formed the basis of this study.

METHODS

We studied twenty-four adult patients in whom spontaneous idiopathic urticaria had been present for more than 3 months. Routine laboratory investigations included a full blood count, ESR, urea and electrolytes, urine microscopy, serum immunoglobulin, protein electrophoresis, rheumatoid factor (SCAT), ANF, DNA binding (where indicated) and Australia antigen.

Biopsies for routine histopathology and immunofluorescence were taken from representative lesions which had been present for less than 12 h. Cases showing deposition of fibrinoid material in the walls of dermal blood vessels were classified as group 1. These cases were associated with a neutrophil-rich perivascular infiltrate and significant leukocytoclasis. They therefore fitted the criteria for the diagnosis of urticarial vasculitis. Cases without evidence of vasculitis were divided into two further groups. Group 2 patients showed a dense perivascular infiltrate which was predominantly mononuclear but contained significant numbers of neutrophils and/or eosinophils. Deposition of fibrinoid material within vessel walls was not seen and leukocytoclasis when present was minimal. Group 3 showed a sparse predominantly mononuclear cell infiltrate and therefore fulfilled the criteria for a diagnosis of ordinary urticaria.

Direct immunofluorescence of lesional skin was performed using commercially available fluorescein isothiocyanate-conjugated antisera to IgG, IgA, IgM, C3 and fibrin (Dako). IMF of clinically uninvolved skin was performed in selected cases only.

Three methods were used for the detection of circulating immune complexes (CICs).

1. *Serum cryoglobulins.* Serum for cryoglobulin analysis was obtained at 37° C and the level evaluated according to Brouet *et al.* (1974). The upper limit of normal for the laboratory was 40 μg/ml.

2. *C1q binding assay (C1qBA).* We used a modification of the methods of Pussell *et al.* (1978). Briefly, serum samples were incubated with ^{125}I-labelled C1q, precipitated with PEG, and the radioactive count determined in the spun precipitate. Results are expressed as a concentration of aggregated IgG giving equivalent precipitation (upper limit of normal 40 μg/ml).

3. *Monoclonal rheumatoid factor binding assay (MrFBA).* Detection of CICs was performed using a modification of the monoclonal rheumatoid factor binding assay (Pussell, unpublished). Briefly serum samples were incubated with ^{125}I-labelled monoclonal IgM kappa extracted from the serum of a patient with esseential mixed cryoglobulinaemia, precipitated with PEG and the

radioactive count determined in the spun precipitate. Results are expressed as a concentration of aggregated IgG giving equivalent precipitation and the upper limit of normal for the laboratory was 40 μg/ml.

Complement studies

Serum complement levels. Total haemolytic complement (CH50) was measured using standard haemolytic plate assays. C3 and C4 were measured by single radial immuno-diffusion using monospecific antisera (Lachman & Hobart, 1978).

Complement conversion products. Factor B and C3 conversion products (C3CP) were measured using cellulose acetate membrane electrophoresis and immunofixation as previously described (Whicher *et al.*, 1980).

RESULTS

The clinical details and routine laboratory tests of the patients are given in Table 1. Lesions from group 1 patients lasted for longer than 24 h and faded to leave slight purpura. In one case bullous lesions associated with intense subepidermal oedema were observed. All group 1 patients complained of arthralgia, but significant renal involvement was not detected. Lesions from group 2 patients sometimes lasted for 24 h, but purpura was not observed. Whilst only one

TABLE 1. Clinical and laboratory findings

Groups	Number	Mean age (and range)	Sex	Duration of disease (months)	Duration of lesions (h)		Arthralgia (No. of pts)	Mean ESR	RhF (SCAT)	ANF 1:10 or greater	Hypergammaglobulinaemia
					> 24	< 24					
1 (Vasculitis)	3	46 (33–61)	3F	18	3	0	3	43	2	3	2
2 (Intermediate)	10	34 (20–59)	6F:4M	10	3	7	7	14	3	5	1
3 (No vasculitis)	11	28 (20–48)	7F:4M	6	0	11	2	9	0	3	0

TABLE 2. Treatment

Group	Piriton 8 mg t.d.s.	Indomethacin 50 mg b.d.	Dapsone 100 mg b.d.	Prednisolone 30 mg o.d.	Plasma exchange
1	NR 0/3	NR 2/2	FR 1/3 PR 2/3	PR 2/2	FR 2/2
2	PR 3/10	D 2/3	NR 5/5	FR 3/3	PR 1/1
3	FR 3/11 PR 5/11	D 2/3	NR 2/2		

FR = Favourable response; PR = Partial response; NR = No response; D = Deterioration.

TABLE 3. Complement levels in group 1

	Patient			Normal range
	(a)	(b)	(c)	
CH50	21	30	70	50–125
C4	12	10	128	50–120
C3	18	35	150	60–135
C3CP	Positive	Positive	Negative	
FBCP	Negative	Negative	Negative	

patient complained of joint pain, six patients admitted to arthralgia during episodes of acute urticaria. Lesions from group 3 patients lasted less than 24 h and only two patients noticed arthralgia.

A strongly positive ANF was found in one group 1 patient only (1 : 160) but DNA binding was within normal range. By contrast a weakly positive ANF (1 : 10) was found in both the other group 1 patients, five group 2 patients and three group 3 patients. Although such levels are not normally considered to be significant in individual patients the incidence of a weakly positive ANF may be of significance between groups of patients.

Treatment is detailed in Table 2. The three groups responded differently. Both group 1 patients who received plasma exchange responded well, but the one patient from group 2 treated with plasma exchange responded only partially. Lesions cleared during the exchange but recurred within 24 h. Oral steroids were used in group 1 and 2 with variable success, but the dosage necessary (prednisolone 30 mg daily) seldom justified their use on a long-term basis. Contrary to previous reports (Millns *et al.*, 1980) indomethacin was generally ineffective in group 1 patients and in groups 2 and 3 it was occasionally associated with clinical deterioration. Antihistamines were generally most effective in group 3 patients whilst dapsone was effective only in group 1 patients. Since the therapeutic effect of dapsone is thought to depend upon its inhibitory effect upon the myeloperoxidase H_2O_2 halide system of the polymorphonuclear leukocyte (Stendhal, Molin & Dahlgren, 1978), its beneficial effect was perhaps predictable in cases characterized by a neutrophil-rich infiltrate.

Table 3 shows the serum complement levels and C3 conversion products in group 1 patients, two of whom showed hypocomplementaemia with evidence of classical pathway activation and C3 conversion. Complement levels for patients in groups 2 and 3 are also shown (Fig. 1). Hypocomplementaemia was not observed but a significant proportion of samples showed raised levels of C3 and/or C4 consistent with an acute phase reaction. C3 conversion products were detected in only two of twenty group 2 samples. Evidence of factor B conversion was not detected.

Positive immunofluorescent findings for the three groups are detailed in Table 4. IgM deposition in lesional and clinically uninvolved skin was observed in two of three group 1 patients. Deposition of C3 and fibrin in lesional skin was seen in groups 1 and 2 whereas group 3 was negative apart from occasional perivascular fibrin fluorescence.

Levels of circulating immune complexes are given in Figs 2 and 3. Apart from one group 3 patient, elevated levels of cryoglobulin were found only in group 1 patients. C1qBA was positive in all group 1 patients, half the group 2 patients and in no group 3 patients. Similar findings were

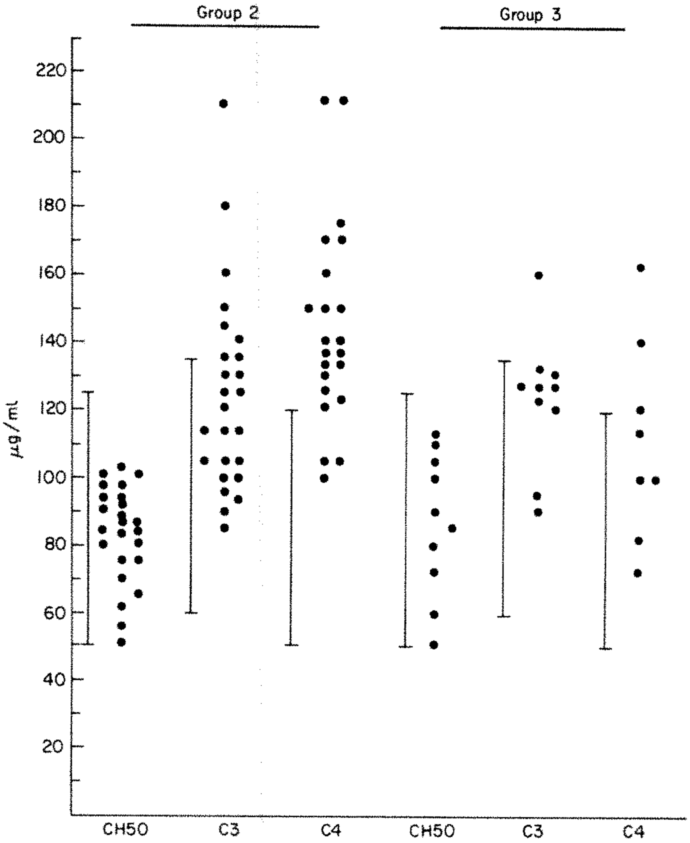

FIGURE 1. Complement levels in groups 2 and 3.

TABLE 4. Direct immunofluorescence

	Group 1		Group 2	Group 3
	Lesional	Non-lesional	Lesional	Lesional
IgM	2/3	2/3	1/10	0/11
C3	2/3	0/3	5/10	1/11
Fibrin	3/3 (vessel wall)	0/3	8/10 (diffuse pattern)	3/11 (diffuse pattern)

obtained with the MrFBA except for one group 1 patient who died before a further serum sample could be obtained.

Fig. 4 shows the relationship between disease activity and C1qBA over a 3-month period during which plasma exchange was carried out at 4- to 6-week intervals. Clearly there is a close association between the two. Towards the end of this period, however, an arterio-venous shunt became infected and although this was associated with a marked rise in the level of circulating

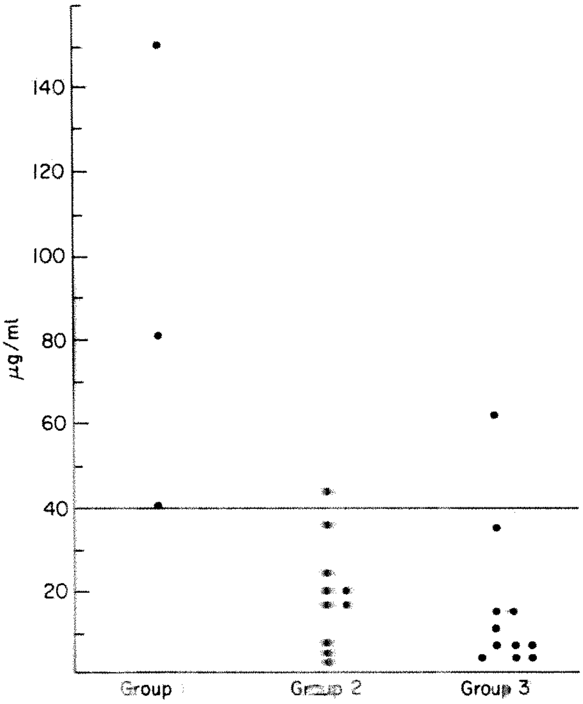

FIGURE 2. Cryoglobulin levels in groups 1, 2 and 3.

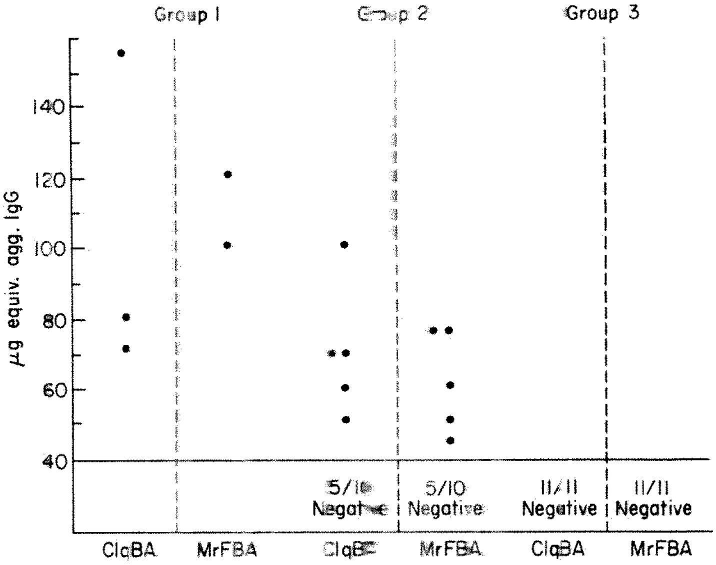

FIGURE 3. Levels of circulating immune complexes in groups 1, 2 and 3.

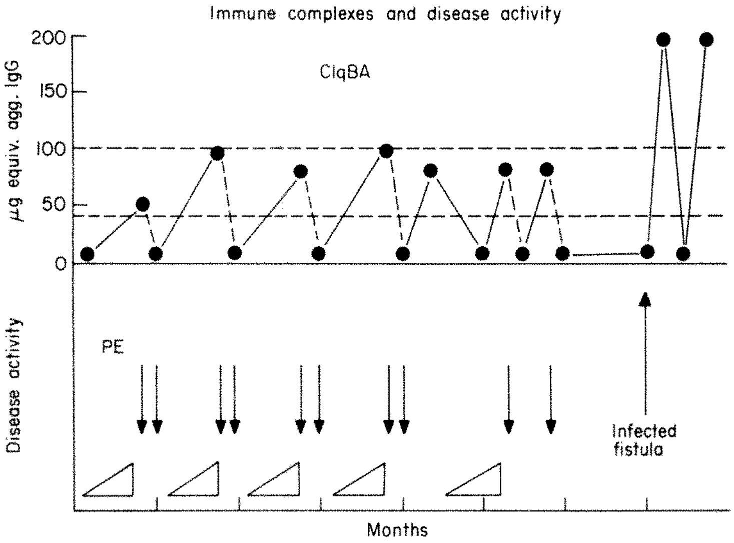

FIGURE 4. Sequential studies in group 1 patient. PE = plasma exchange.

immune complexes it was not associated with any disease relapse. These observations suggest that the C1qBA may detect more than one type of complex in the same patient.

DISCUSSION

The diagnosis of urticarial vasculitis rests upon the histology and these patients are further distinguished from those with ordinary urticaria by the immunopathological changes. The present study confirms this, but also demonstrates the existence of a further group of patients with features intermediate between ordinary urticaria and urticarial vasculitis. Although the classification is based on histopathological criteria, the clinical and immunopathological features also help to distinguish these three groups.

Group 1 patients were characterized by small amounts of circulating cryoglobulin, elevated levels of circulating immune complexes and evidence of classical pathway complement activation in two out of three cases. Direct IMF studies demonstrated deposition of IgM (2/3), fibrin (3/3) and C3 (2/3) within dermal vessels. In addition two cases showed linear deposition of IgM at the basement membrane zone in both clinically involved and uninvolved skin.

Group 3 patients did not exhibit such features apart from occasional perivascular fibrin fluorescence (3/11 cases). IMF of lesional skin was negative and serological tests revealed no evidence of circulating immune complexes or hypocomplementaemia.

Group 2 demonstrated intermediate features. Although elevated levels of circulating immune complexes were detected in half the cases, hypocomplementaemia was not present and evidence of complement conversion was found infrequently. By contrast, deposits of C3 within or around dermal blood vessels were found in 5/10 cases and fibrin in 8/10 cases. These changes were, however, confined to lesional skin.

These findings should be compared with the study by Monroe *et al.* (1981) where a similar spectrum of histopathological and immunopathological changes were reported. It seems likely, however, that our criteria for a diagnosis of vasculitis were somewhat stricter. In our experience cases of urticarial vasculitis are uncommon. At St John's Hospital only three cases were diagnosed in the course of a year (out of 17,000 new referrals). By contrast Monroe *et al.* (1981) identified nine cases in a prospective study of forty-five patients with chronic urticaria. It is possible that some of Monroe's vasculitis group would have been included as group 2 patients according to our criteria. The relatively low incidence of arthralgia, circulating immune complexes and positive-IMF results in his vasculitis group and the fact that only one of his nine patients exhibited hypocomplementaemia provide some support for this interpretation. Otherwise our two studies are complementary and show remarkable similarities.

Urticarial vasculitis, like other varieties of cutaneous leukocytoclastic vasculitis, involves post-capillary venules and is widely regarded as an example of a type 3 hypersensitivity reaction. The presence of circulating immune complexes, hypocomplementaemia and immunoreactant deposition in lesional skin demonstrated in this and other studies supports the idea of a causal relationship between the serological findings and the pathological changes. Furthermore it is generally argued that these features help to distinguish urticarial vasculitis from ordinary urticaria. However, this method of classification is complicated considerably by our cases in group 2. Should one regard such cases as a mild variant of urticarial vasculitis, or a florid variety of urticaria?

The relationship between CICs and disease processes could operate in any of three ways. The orthodox view is that the deposition of CICs in blood vessel walls initiates the inflammatory response. A second view is that CICs are an invariable accompaniment of disease processes and result from the preceding tissue inflammation. A further possibility is that they are indirectly associated through the interaction of a third variable, such as compromised reticulo-endothelial function, which might contribute to the inflammation and the raised levels of CICs. To date few studies have attempted to unravel these possibilities.

We gained some insight into these relationships by carrying out sequential studies in individual patients using plasma exchange as a therapeutic tool. Fig. 4 documents a clear correlation between C1q binding and disease activity in a group 1 patient. Plasma exchange removed C1q binding activity and was followed by a period of disease remission which lasted from 4–6 weeks. These findings are consistent with the hypothesis that CICs cause the cutaneous lesions, but they do not exclude other possibilities. This latter consideration is certainly relevant since reticulo-endothelial function (as measured by the half-life of [^{51}Cr]IgG-coated red blood cells) was known to be compromised in this patient and was also reversed by plasma exchange.

In experimental immune complex disease the influence of genetically controlled factors such as antibody level and antibody affinity will determine the size and properties of the resulting CICs (Devey & Steward, 1980; Devey *et al.*, 1982). Similar factors may explain some of the immunological findings in our study. Clearance of CICs may also be important and could account for the different level of CICs and the different response to plasma exchange observed in groups 1 and 2. Since there is some evidence that reticulo-endothelial function is itself HLA-dependent, future studies may well identify HLA typing as a further method of distinguishing these 3 groups.

We conclude that patients with urticaria show a spectrum of disease activity and that this is expressed in both the histopathological and the immunopathological findings.

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Sweat gland disturbances in granuloma annulare and necrobiosis lipoidica

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SUMMARY

We studied sweat gland distribution, density and activity in thirteen cases of granuloma annulare and ten cases of necrobiosis lipoidica, using a combination of the plastic impression and starch-iodine methods. The pattern of sweat gland disturbance in the two diseases was entirely different. In necrobiosis lipoidica an intense and uniform hypohidrosis was detected throughout the lesion, whereas in granuloma annulare the disturbance followed the morphology of the lesion (the papular border showed complete anhidrosis, whereas the flat central part of the lesion showed only moderate hypohidrosis or normal sweating). The method assigns numerical values to the 'relative density' and the 'relative activity' of the functioning sweat glands compared with normal skin, thus permitting statistical evaluation of the results.

The disturbances in sweating in granuloma annulare (GA) and in necrobiosis lipoidica (NL) were studied by combining the plastic impression and the starch-iodine methods, and a method was devised for numerical comparison of the results.

METHODS

The sweat gland disturbances in thirteen cases of GA (six male and seven female, aged 3–66 years, mean age 32·8 years), and ten cases of NL (all female, aged 35–64 years, mean age 50·4 years) were studied. The diagnosis in every case was confirmed histologically. All the experiments were done during the summer season.

Heat-provoked sweating was detected by the plastic impression technique in combination with the starch-iodine method (Hatzis, 1978) as follows: the area to be studied was painted with a 5% non-alcoholic iodine solution (because alcohol can cause anhidrosis; Papa, 1963) and after about one minute, when the area had dried well, starch powder was applied in a very thin layer with the aid of cotton wool. Immediately after that, the silicone material ('Silaplast', Dental Karl Huber Kg., 75 Karlsruhe, W. Germany) was mixed well with a few drops of catalyst (4 drops of

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catalyst to 8 ml of silicone), and the area for examination was covered with it. After about 20 min, on removing the imprint, the sweat droplets could be seen and studied on the white area of silicone material, which showed a cyclic or oval shape with a deep violet stain.

The healthy skin surrounding the lesion was used as control, instead of the corresponding symmetrical area opposite. We believe this to be a more precise control, because of the asymmetry in sweating which exists in normal people (Herrmann, Prose & Sulzberger, 1952; Montagna & Parakkal, 1974).

We studied the distribution, density and activity of the functioning sweat glands as follows, using overhead projection of the replica on a screen:

(a) the topographical distribution of the sweating in the area of the lesion. This may be uniform throughout the lesion, or variable, in which case it may or may not relate to the morphology of the lesion.

(b) the density of the functioning glands in the lesion compared with the normal. This was expressed as 'relative density' (r.d.) and defined by dividing the number of functioning glands per cm^2 (density) in one area of the lesion, by the density of the functioning glands in the adjacent healthy area (control), multiplied by 100.

In normal skin, the percentage of functioning glands depends on the individual's sex and age, the site studied, the climate and the conditions during the test itself (Rothman, 1954; Grice & Verbov, 1977). Given that this phenomenon is under central nervous control (Rothman, 1954), the functioning glands in the area of a skin lesion should also be subjected to the same control. Thus the r.d. should remain constant, independent of various factors which may affect the number of functioning glands.

(c) the rate of sweat production in the functioning glands in the lesion compared with the normal. This was expressed as 'relative activity' (r.a.). Thus the r.a. is calculated by dividing the area of a certain number of sweat-droplet impressions in the lesion, by the area of an equal number of impressions in the healthy skin (control), multiplied by 100. Because of the circular shape of sweat droplets the area (πr^2) for one sweat gland impression is proportional to the rate of sweat production. The sum of the droplet areas from one region of skin indicates the rate of sweat production for that region, and this represents the sweat glands' functional intensity. Thus by comparing the sum of the impression areas from the same number of glands in a lesion and normal skin, their ratio will measure the rate of sweat production in the lesion with respect to normal.

Thus $$\text{r.a.} = \frac{\sum^{n} \pi r^2 \text{ lesion}}{\sum^{N} \pi R^2 \text{ normal}} \times 100$$

The r.a. gives the percentage increase or decrease of the excretion rate for the functioning glands. This ratio is preferable to the mean value, since the frequency distribution of impression areas may be totally different in normal and lesion samples. The r.d. and r.a. may be calculated for the entire lesional area or a special part of it.

RESULTS

Necrobiosis lipoidica

Sweating was uniformly decreased throughout the lesion because the few functioning glands were sporadically distributed (Fig. 1). The r.d. and r.a. values represented the entire lesional area and they showed marked hypohidrosis (Table I). Independent of the clinical picture, time

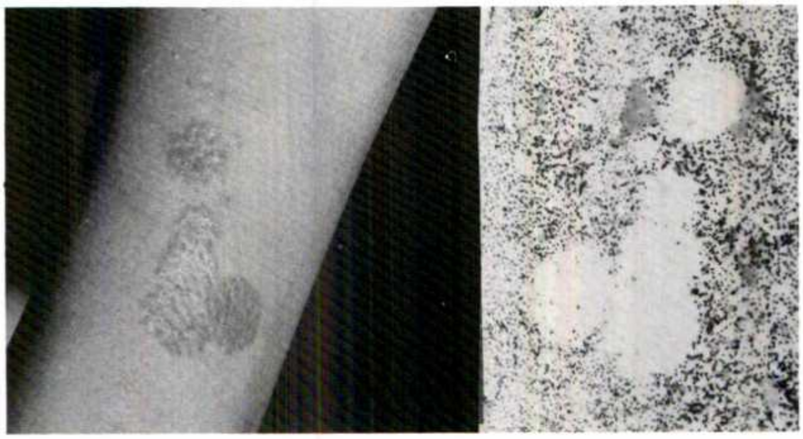

FIGURE 1. Sweat gland activity in necrobiosis lipoidica.

TABLE 1. Sweat gland activity in necrobiosis lipoidica and granuloma annulare

	Mean r.d. ± s.d. %	Mean r.a. ± s.d. %
Necrobiosis lipoidica site:		
The whole lesion	7 ± 7	15 ± 15
Range of normal values (n = 10)	93–107	89–111
Granuloma annulare site:		
(a) papular border	0	0
(b) centre of lesion	76 ± 20	77 ± 22
Range of normal values (n = 13)	94–106	87–113

r.d. = Relative density, r.a. = Relative activity, s.d. = Standard deviation.

of appearance (2–10 years), position of the lesion, coexistence of diabetes or not (50% presented diabetes) and age, out of the ten NL cases, seven showed severe hypohidrosis, whereas the remaining 3 presented anhidrosis.

Granuloma annulare

The distribution of sweat disturbance tended to follow the morphology of the GA lesion. More precisely, the papular periphery or the isolated GA papules showed complete anhidrosis. However the central part of the affected area which had begun to flatten or was already levelled, showed characteristic activity which varied from moderate hypohidrosis to normal sweating (Fig. 2, Table I). This form of sweating was observed in all thirteen cases of GA, independent of the clinical picture (this pattern was also observed in one case of subcutaneous GA), time of appearance (3 months–6 years), position of the lesion, sex, age and coexistence of diabetes (23%

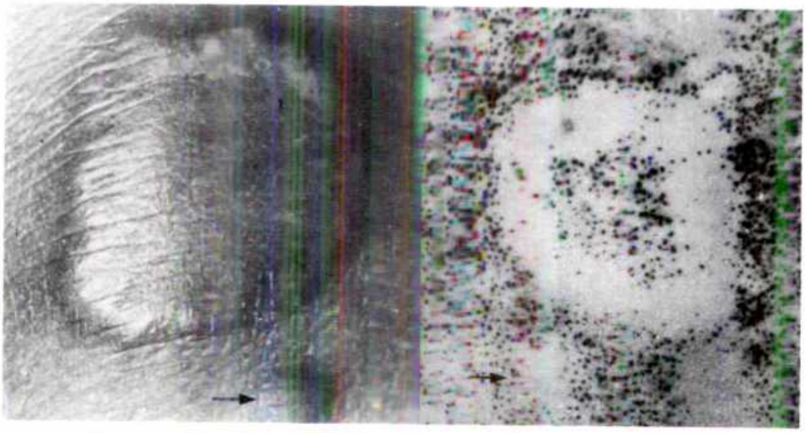

FIGURE 2. Sweat gland activity in granuloma annulare. Arrow shows an isolated papule.

presented diabetes). This characteristic type of sweat pattern depended only on the size of the lesion, which we found had to be greater than 7 mm in diameter. The r.d. and r.a. for GA were calculated separately, in the papular border and in the levelled area of the lesion (Table 1).

When there were few functioning glands in the lesion (e.g. ten) there were problems in calculating the r.a. In this example we can calculate the sum of 100 adjacent droplets in the normal ($\sum_1^{100} \pi R^2$ normal), this is divided by 10 and the result is the denominator of the sum of the ten sweat stigmata in the lesion. So the r.a. formula in this case would be

$$\text{r.a.} = \frac{\sum_1^{10} \pi r^2 \text{ lesion}}{\sum_1^{100} \pi R^2 \text{ normal} \div 10} \times 100.$$

We encountered this problem in calculating the r.a. in two cases of NL.

DISCUSSION

Since it was shown that the sweat gland disturbances in GA and in NL have entirely different patterns (Figs 1 and 2), it may be possible to use this test as an aid in distinguishing these two diseases.

It should be noted that fluctuations in the density (or activity) of the functioning glands were observed in the adjoining square cm even in the lesional area and in the normal skin which acted as control. To overcome this discrepancy, the number of functioning glands in the normal or in the lesional skin was therefore taken to be the median of the minimum and the maximum values which we obtained by measuring the density of the functioning glands in four contiguous square cm. The density of functioning glands in the normal skin, in all the cases, was 55–120 per cm^2. The median value of the activity was calculated in the same manner. At the same time, the normal percentage fluctuation was also recorded (Table 1).

The sweat gland function is periodical, under central control. The number of active and resting sweat glands, at any one time, depends on the environmental conditions, on the body region and on the individual (Rothman, 1954; Groce & Verbov, 1977). Willis, Harris & Moretz (1973), studying the sweat gland function under thermoregulatory conditions and after pilocarpine iontophoresis, divided the functioning glands of the normal skin, according to the size of their impression on the imprint, into 'maximally functioning' and 'poorly functioning'. We have observed similar figures, but the above characterization of sweat glands can only be used in pharmacological sweating, and it is not successful in experiments based on nervous sweating. This is because the size of imprint impression (representing the amount of sweat) in the case of nervous sweating depends not only on the functional level of the gland, but also on its phase of function during the time of experiment. The functional phase of the glands in two small adjacent regions must be similar, because of similarity in function frequency. So differences in the sum of an equal number of the areas of impressions between two small adjacent regions (i.e. normal to normal or lesion to normal regions), will represent functional differences in sweat gland activity between the two small regions. That is because all the other factors (frequency of function, phase of function, time of experiment and experimental conditions) are similar or constant. This is expressed by the r.a. value.

The advantage of the r.d. and r.a. values is that they give a direct picture of the state of sweating in the lesion for easy comparison, and they also allow statistical evaluation of the results. The r.d. value is of more importance than the r.a., because it represents the complete suppression of some or all of the sweat glands.

The combination of the plastic impression technique, with the starch-iodine method (Hatzis, 1978) has greatly simplified this procedure in comparison with previous similar techniques (Sarkany & Gaylarde, 1968; Harris, Polk & Willis, 1972; Willis *et al.*, 1973). In addition this modification allowed us to follow the course of the sweat gland activity during the experiment (looking under the edges of the imprint material), thus enabling us to remove the imprint at the appropriate time. The only disadvantage of this modification is that due to the evaporation of the iodine; the impression is only suitable for study for approximately 1 week after its preparation. Thus if records are to be kept, the samples must be photographed.

ACKNOWLEDGMENT

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Case Reports

Merkel cell tumour: report of a case

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SUMMARY

We report the case of a 75-year-old lady with recurrent primary cutaneous Merkel cell tumours. The condition can be misdiagnosed as lymphoma or anaplastic carcinoma, and the definitive diagnosis can be made only by electron microscopy of fresh tissue. This is the first reported case in the British literature.

In 1875 Merkel described an epidermal cell which was innervated by myelinated nerve fibres and functioned as an end organ for touch sensation (Merkel, 1875). Merkel cells are now classified as slowly adapting Type I mechanoreceptors (Sinclair, 1981). They are found in the Hederiform endings of Ranvier, the Pinkus corpuscle and occasionally free in the dermis. Ultrastructural studies show numerous cytoplasmic dense core granules, 70–90 nm in diameter, polarized towards the terminal neurone (Winkelmann & Breathnach, 1973). These granules are similar to those found in neuroepithelial and neurosecretory cells but so far immunohistochemical techniques have failed to define their contents. It is thought that they are involved in the function of the cell as a transducer of mechanical to quantal chemical or electrical activity. Winkelmann (1977) postulated that Merkel cells belong to the APUD series because (1) the cells are closely apposed to terminal neurones, (2) the cells contain neurosecretory granules and (3) they are thought to migrate from the neural crest. Since their derivation from the neural crest is unproven, their inclusion in the APUD cell series remains hypothetical (Pearse, 1980).

CASE REPORT

In 1979 this 75-year-old female presented with a slowly growing tumour on the left knee and two similar lesions on the right shin. These tumours were excised in August 1980 and histology at another hospital showed changes consistent with metastatic deposits of an undifferentiated, highly malignant tumour in the subcutaneous fat and dermis. A carcinoma of the bronchus or an undifferentiated lymphoma were erroneously considered as the most probable primary sites. Examination and extensive investigations were normal. In October 1980, three small satellite nodules developed near the previously resected tumours on the right shin. By April 1982 they had enlarged to form two tumours one measuring 9 × 8 × 4 cm on the medial aspect and one

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5 × 4 × 2 cm on the lateral aspect of the right lower leg (Fig. 1). They were painful and bled after minor trauma. Examination was otherwise normal and there was no lymphadenopathy.

The following investigations were normal or negative: urea and electrolytes, liver function tests, calcium, phosphate, plasma adrenaline and noradrenaline, plasma parathormone, plasma insulin and growth hormone, plasma neurotensin, gastrin and vasoactive inhibitory polypeptide, carcinoembryonic antigen and beta human chorionic gonadotrophin. Plasma calcitonin was raised at 0·22 μg/l (normal range $< 0{\cdot}08$), and the α-fetoprotein was 14 U/ml (normal range < 10). A skeletal survey showed a fracture of the right ninth rib only but a bone scan showed

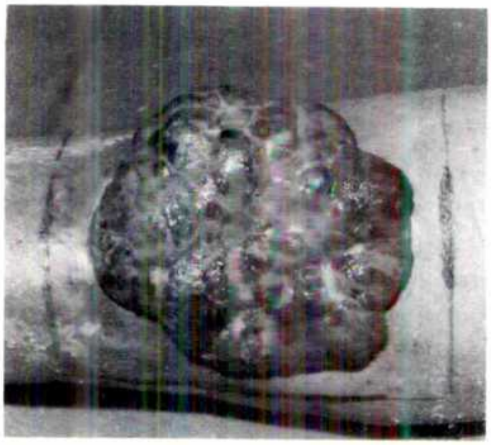

FIGURE 1. Tumour on medial aspect of right lower leg above the ankle.

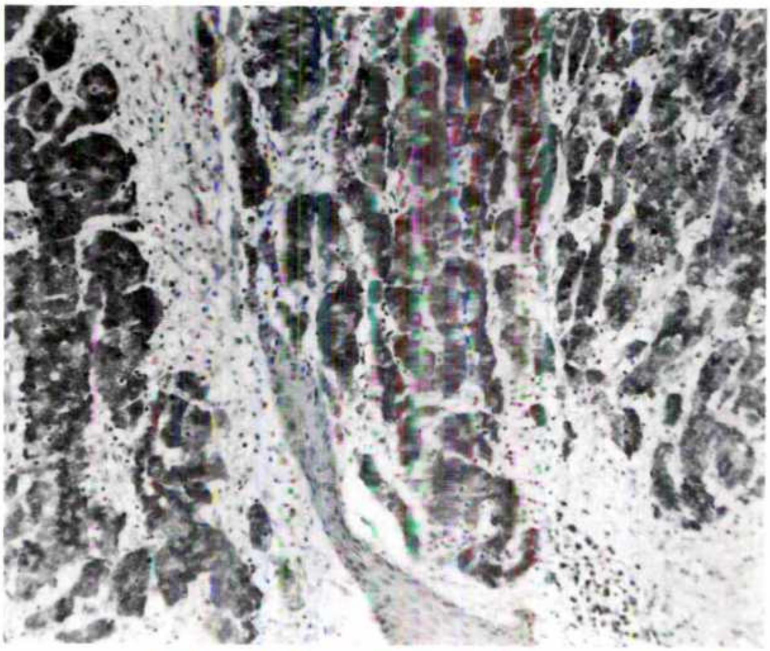

FIGURE 2. Sheets of darkly staining cells forming clumps and trabeculae beneath epithelium (H & E, × 90).

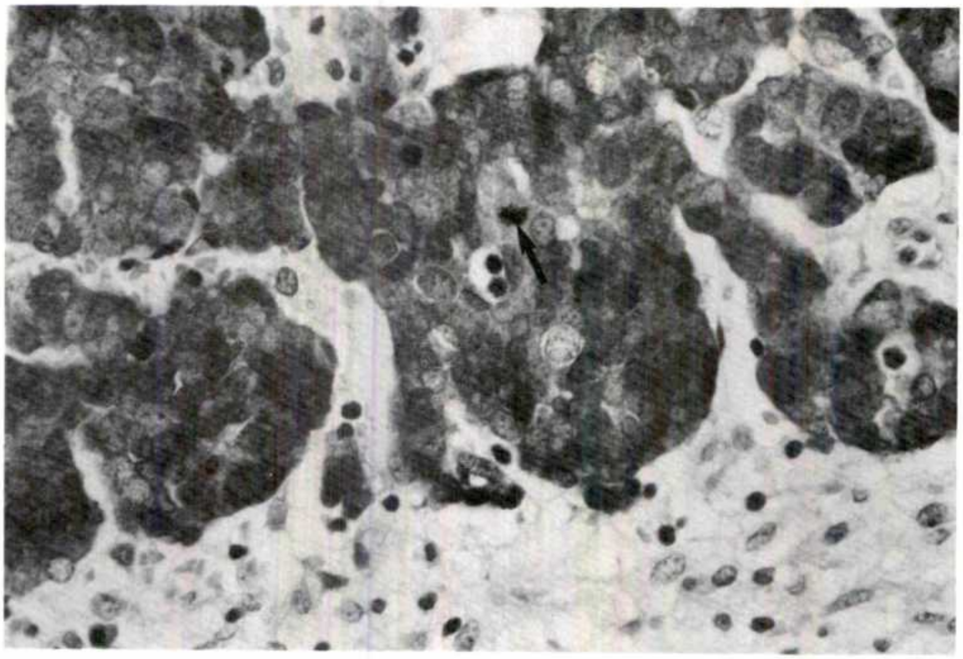

FIGURE 3. Cohesive masses of hyperchromatic cells with mitosis (arrow), (H & E, × 300).

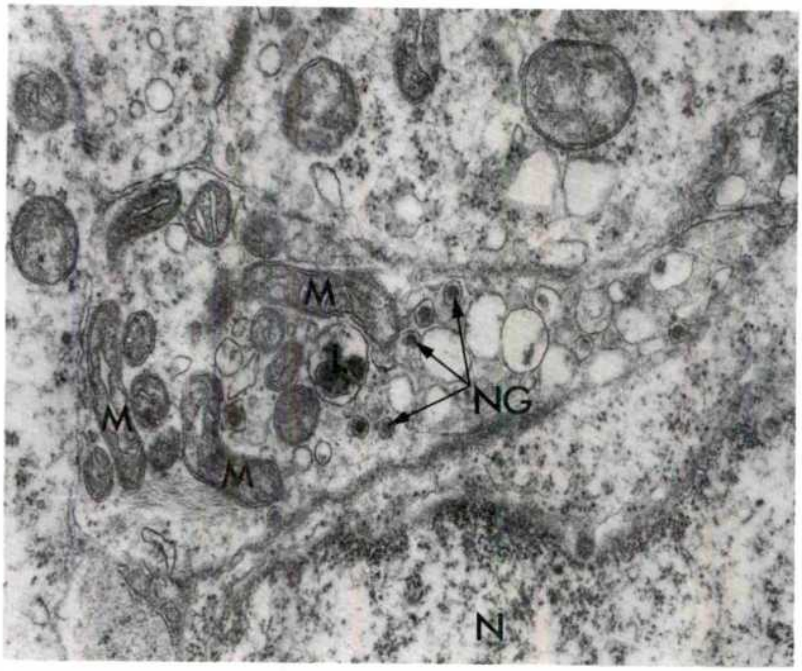

FIGURE 4. Electron micrograph of tumour cell showing nucleus (N), lysozome (L), scattered mitochondria (M) and numerous cytoplasmic neurosecretory granules (NG) (× 40,000).

increased uptake in several ribs on the right, both knees and medial malleolus of the right ankle. These changes were compatible with metastases, but because they were asymptomatic a bone biopsy was not performed. A CT scan of the abdomen and a thyroid scan were normal.

A biopsy examined under light microscopy showed cells with large nuclei and scant cytoplasm arranged in clumps and trabeculae (Figs 2 and 3). Numerous mitoses were present. Despite the large proportion of mitotic figures, the cells were of relatively uniform size and shape, and the appearances were unlike those of a melanoma. Furthermore, a Masson Fontana stain was negative. The relative cellular uniformity and trabecular pattern suggested that this tumour might be a Merkel cell tumour. This was supported by the presence of numerous cytoplasmic neurosecretory granules in electron microscopic preparations (Fig. 4). Immunohistochemical staining for calcitonin, somatostatin, bombesin, substance P, neurotensin, metenkephalin, beta LPH and alpha HCG were all negative. Review of the 1980 biopsy showed the same light microscopy findings but no suitable tissue was available for electron microscopy.

The tumours were treated with radiotherapy, 60 Gray given over 40 days in twenty-four fractions. They regressed in size but the field was increased because of a local recurrence. Further local recurrences appearing within 6 weeks of commencing treatment have been treated with radiotherapy.

DISCUSSION

In 1972 Toker described five cases of 'trabecular carcinoma' of the skin which in retrospect were probably of Merkel cell origin. The tumours, which were located in the dermis, were composed of solid trabeculae of cells. They had numerous mitoses and metastasized to lymph nodes. The histological features were similar to those of highly malignant undifferentiated neoplasms but their biological behaviour was of a low grade malignancy. Tang & Toker (1978) reported on three similar neoplasms which ultrastructurally had neurosecretory granules in the cytoplasm. Merkel cells and melanocytes are the only cells in the skin normally containing neurosecretory granules. Since the latter can readily be distinguished by the presence of melanosomes and a positive DOPA reaction, they concluded that these neoplasms were derived from Merkel cells.

Merkel cell tumours usually present as cutaneous nodules and their rate of growth is variable. Local recurrence occurs in approximately 50% of patients after surgical excision or radiotherapy and they commonly metastasize to regional lymph nodes and occasionally to distant sites (Table 1). Five patients from a total of thirty-four reported in the literature have died with disseminated disease.

TABLE 1. Recurrence and metastasis of Merkel cell tumours in thirty-four patients

		No. of patients	Local recurrence	Nodal metastasis	Distal metastasis	Death
Toker	1972	5	2	3	2	1
Tang & Toker	1978	3	—	1	1	—
Sibley *et al.*	1980	2	2	2	1	1
Johannessen & Gould	1980	1	1	1	—	—
Wolf-Peeters *et al.*	1980	5	2	1	1	—
Sidhu *et al.*	1980	7	3	4	—	—
Silva *et al.*	1980	11	3	6	3	3
		34	13	18	8	5

This patient had a raised plasma calcitonin of 0·22 μg/l on presentation and this fell to 0·15 μg/l (normal range < 0·08) after radiotherapy to the tumours. There has been one other report of a Merkel cell tumour being associated with a raised calcitonin (Johannessen & Gould, 1980). In this case, calcitonin levels remained elevated after thyroidectomy for a suspected C-cell tumour and only returned to normal after a metastasis from the Merkel cell tumour was removed. Calcitonin levels again became elevated when further metastases developed. In our patient, the source of the calcitonin is uncertain since immunohistochemistry of tumour tissue was negative and a thyroid scan was normal. Serial calcitonin levels could be used as a tumour marker and this feature is consistent with the functional characteristic of an APUD cell to produce peptide hormones.

The true incidence of Merkel cell tumours is not known because they can be misdiagnosed as cutaneous lymphomas or undifferentiated neoplasms by light microscopy. A definitive diagnosis can be made only by electron microscopy of fresh biopsy material rather than routinely processed tissue and as yet this technique is not routinely used for morphological diagnoses (Olsen, 1982).

ACKNOWLEDGMENT

We wish to thank Juliet Pettitt for the electron microscopy.

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A study of familial lupus erythematosus-like disease and hereditary angio-oedema treated with danazol

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SUMMARY

Immunological and genetic studies were performed in a family in which the mother and five siblings had hereditary angio-oedema (HAE). LE cells, antinuclear factors, antibodies to double-stranded DNA and positive direct Coombs' test were not found in any of the subjects. One female sibling had a lupus erythematosus-like illness with skin lesions of the face which responded to danazol treatment. Laboratory evidence of HAE was obtained in the entire kindred and all the siblings shared HLA haplotype A1B7 inherited from the deceased mother.

Hereditary angio-oedema (HAE) is an inherited disorder of the complement system caused by the decreased synthesis (Donaldson & Evans, 1963) or functional deficiency (Rosen *et al.*, 1965) of the inhibitor of activated first complement component (C1-INH) and manifested by repeated attacks of angio-oedema involving the skin, gastro-intestinal tract and larynx. Until recently the prognosis was extremely poor, but effective prevention of attacks is now possible with the administration of danazol, an ethinyltestosterone derivative with markedly attenuated androgenic potential (Gelfand *et al.*, 1976). About 2% of patients with HAE (Donaldson, Hess & Adams, 1977; Shiraishi *et al.*, 1982) have concurrent lupus erythematosus-like illness (LELI). In a preliminary report, we described the first case of reversal of LELI with danazol (Masse *et al.*, 1980; Donaldson & Hess, 1980). We now present the results of immunological and genetic studies in this patient's family (Fig. 1) and the results of treating the entire kindred with danazol.

CASE REPORTS

Propositus (II-4)

The patient was a 24-year-old woman with a 2-year history of episodic attacks of abdominal pain. On admission, there was an extensive scaling rash on the face and severe solar sensitivity.

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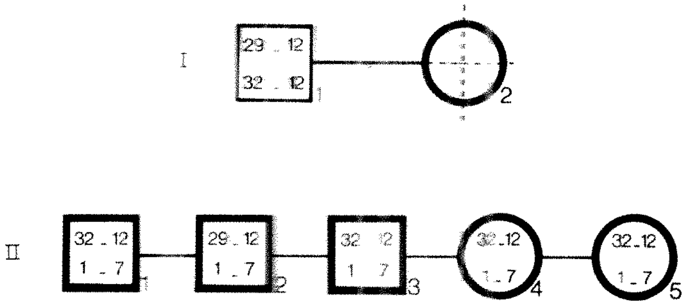

FIGURE 1. Pedigree of family studied. Symbols in heavy outline denote hereditary angio-oedema. Squares refer to males, circles to females. The mother is deceased. HLA haplotypes are shown within the symbols.

The patient could recall no bouts of fever or joint pain over the previous year. No other physical abnormalities were seen. Urinalysis, blood urea and serum creatinine were normal. Biopsy of a skin lesion gave findings compatible with a diagnosis of subacute lupus erythematosus, viz. partial epidermal atrophy and a moderate inflammatory infiltrate around the follicles. Immunofluorescence microscopy showed no IgG, IgA, IgM or C3 deposition. The patient was put on danazol, 200 mg daily, for 6 months. The skin lesions disappeared within 4 weeks. After the initial 6-month period, danazol was given on an alternate day regimen and after 8 weeks, treatment was discontinued. Four weeks later the facial rash recurred and responded to a second course of danazol.

Father of Propositus (I-1)
The father showed no clinical or laboratory evidence of disease.

Mother of Propositus (I-2)
The mother first developed angio-oedema of the face at the age of 16. Subsequent attacks of cutaneous and laryngeal oedema occurred every 2 to 4 weeks, lasting each time from 12 to 18 h. She had five pregnancies, in the course of which the attacks of oedema increased in frequency. At the age of 32 she died from laryngeal oedema.

Sibling II-1
An attack of cutaneous angio-oedema and colicky abdominal pain first occurred at the age of 13 in this male sibling. Attacks lasted from 24 to 48 h and recurred about every 2 weeks. At the age of 17, danazol was started in a daily dose of 600 mg. This regimen was continued for 9 months, followed by 400 mg daily for 2 years and currently 200 mg daily. Since danazol treatment was started no further attacks have occurred.

Sibling II-2
This male sibling is apparently in good health and has no history of cutaneous angio-oedema or joint symptoms. His only complaint is periodic abdominal pain. Current prophylactic treatment consists of 200 mg of danazol daily.

Sibling II-3
Peripheral swelling and abdominal cramps with vomiting first occurred at the age of 3 in this male sibling. Over the next 20 years no further symptoms occurred. At the age of 24 he was

submitted for emergency abdominal surgery. At the operation, retro-peritoneal oedema was seen to affect the entire coeliac region, infiltrating the mesentery, mesocolon and duodenal wall. Current treatment consists of 200 mg of danazol daily and the patient is symptom-free.

Sibling II-5

At the age of 8 this female sibling experienced an attack of facial angio-oedema. Over the next 2 years attacks recurred about every 2 weeks. Between 10 and 15 years of age she was symptom-free. At the age of 16, attacks of abdominal pain and vomiting occurred each week. Over the past year she has been taking danazol, 200 mg on alternative days, and has experienced no further attacks.

METHODS

Standard haemolytic titration of total serum complement (CH 50 U/ml) was performed by the method of Mayer (1961) and of C1, C4, C2 and C3 by the method of Hartmann & Brecy (1969). The results were expressed as percentages of mean values for serum samples from forty healthy blood donors. Assay of C1-INH was based on measurement of the ability of the patient's serum to block the esterolytic activity of a standard amount of C1 esterase on *N*-acetyl-L-tyrosine-ethyl-ester. Protein concentrations of C1-INH, C4, C3 and B factor were determined by single radial immunodiffusion using commercially prepared plates (Behringwerke). Immunochemical estimation of C1q was performed by radial immunodiffusion using antiserum purchased from Behringwerke. Immunodiffusion data were compared with normal values obtained from fifty-nine healthy volunteers. Antinuclear factor (ANF) was tested by indirect immunofluorescence (IIF) using rat liver substrate and fluorescein-isothiocyanate-conjugated goat antiserum against human immunoglobulin (Meloy). Antibodies to double-stranded deoxyribonucleic acid (dsDNA) were determined by the [^{14}C]DNA-binding assay (Amersham) and by IIF using *Crithidia luciliae* substrate. Direct Coombs' test (DCT) was performed in all the subjects. HLA-A and -B locus antigens were typed by a standard lymphocyte microcytotoxicity method using fifty-eight sera (France-Transplant) defining ten A locus and sixteen B locus specificities.

RESULTS

Measurements of C1-INH and haemolytic titrations of total serum complement (5·07 ± 1·26 units/min/ml and 137 ± 37 units/ml, respectively, in forty blood donors) and its components

TABLE 1. Complement profiles of kindred before danazol treatment. All values are percentages of normal means in forty blood donors

Patient	C1-INH	CH50	C1	C4	C2	C3
II-1	0	65	55	14	34	77
II-2	0	65	52	45	49	70
II-3	0	60	73	59	74	88
II-5	0	58	42	11	41	74

C1-INH = inhibitor of activated first complement component.

TABLE 2. Complement profiles of propositus before and during danazol treatment. All values are percentages of normal means (forty blood donors for enzymatic and haemolytic titrations; fifty-nine healthy volunteers for radial immunodiffusion technique—with asterisk)

Date	C1-INH	CH50	C1	C4	C2	C3	Bf
Before danazol							
Feb 1975	0	26	80	16	11	74	ND
Mar 1979	0	66	102	23	43	43	ND
During danazol							
Apr 1980	25*	94	77	7*	ND	108*	129*
Oct 1980	0	62	94	33	54	88	ND

Bf = factor B; ND = not done.

performed before danazol treatment are shown in Table 1. In the five siblings C1 INH activity was absent, C4 and C2 gave very low values and total serum complement and serum C3 were near normal.

Serial complement assays performed in the propositus (II-4) before and during danazol treatment (Table 2) showed no increase in C1-INH despite clinical response to treatment. Siblings II-1, II-2, II-3 and II-5 were not available for repeated assays but the clinical response to danazol treatment was striking in all four. In all siblings, tests for ANF and anti-dsDNA antibody and DCT were repeatedly negative.

HLA typing data are shown in Fig. 1. All the siblings shared haplotype A1B7 transmitted by the mother. It was not known whether she was homozygous or heterozygous for this.

DISCUSSION

LELI or focal glomerulonephritis (Spitzer, Stitzel & Urmson, 1980) can occur in patients with low serum concentration or functional deficiency of C1-INH (Donaldson *et al.*, 1977) and has been described in association with genetic defects of components involved in the classic complement reaction sequence, viz. C1q (Thompson *et al.*, 1980), C1r (Moncada *et al.*, 1972), C1s (Pondmann, Stoope & Cormaine, 1968), C4 (Haupmann *et al.*, 1974) and C2 (Agnello, de Braco & Kunkel, 1972). The drastic reduction in C4 and C2 levels in the absence of C3 depletion is probably due to their excessive consumption in HAE. Indeed, a certain degree of sustained spontaneous activation was in evidence in the five siblings of the present series. However, low C4 has been shown to be related to combined C4 hypercatabolism and decreased synthesis (Carpenter *et al.*, 1969). One possibility is that impairment of immune utilization of putative virus-antibody complexes could increase vulnerability to latent viral infection and thus explain the association of LELI with HAE. Another possible mechanism underlying the pathogenesis is is deficient direct viral elimination. The likelihood of proliferation of such an agent may be enhanced in some deficient individuals.

Response to danazol treatment was striking in the five siblings. Following onset of treatment all were symptom-free. In four of the five, complement titres during treatment were not measured but a rise in C1-INH and C4 during treatment to near-normal levels is well documented (Gelfand *et al.*, 1976). A low dose regimen of 200 mg every other day was sufficient to maintain an 8-year-old boy symptom-free and to raise C1-INH and C4 to half-normal levels (Tappeiner *et al.*, 1979). The propositus of the present family exhibited a clinical improvement in the absence of laboratory response. This patient had a picture of LELI, which does not fulfil the American Rheumatism Association's criteria for a diagnosis of systemic lupus erythematosus. She was negative for ANF and DCT, as were her siblings. Her skin lesions disappeared within 4 weeks of the start of danazol treatment and recurred 4 weeks after stopping treatment. The reported failure to suppress autoimmunity in NZB/NZW mice with danazol (Roubinian *et al.*, 1979) suggests that the beneficial effect of this drug in HAE might be the result of an anabolic mechanism rather than of its androgenic properties. This might also be true of other androgens such as 5-alpha-dihydrotestosterone.

HAE is known to be transmitted as an autosomal dominant trait with incomplete penetrance (Ruddy & Austen, 1970). The family presented here is noteworthy on two counts. First, all the siblings were affected; second, all shared haplotype A1B7. Unfortunately, the mother's parents were not available for examination. These two facts suggest genetic linkage between the HLA system and the HAE. The occurrence of A1B7 haplotype in each of the affected siblings is most likely to be a chance occurrence since there is considerable evidence that there is no linkage between the HLA system and HAE (Jackson *et al.*, 1978).

Clearly, the potential lethality of HAE makes it imperative to perform complement studies on every patient with lupus erythematosus skin lesions and all available family members.

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Acute urticaria due to alcohol

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SUMMARY

A patient with urticaria caused specifically by alcohol is described. The reaction could be blocked by indomethacin and naloxone, indicating that the eruption was mediated by endogenous opiates and prostaglandins. Sodium cromoglycate blocked the reaction completely and consistently.

CASE REPORT

A 36-year-old man presented in June 1981 with a 3-month history of acute urticaria. The episodes occurred invariably and exclusively after drinking a small amount of alcohol, for example one glass of sherry or one pint of beer. There was no history of associated flushing of the chest or face. His general health was excellent and his past medical history was unremarkable. He was taking no medication.

Oral challenge tests with tartrazine, 4-hydroxy-benzoic acid, sodium benzoate, brewer's yeast, penicillin and aspirin produced no reaction. Provocation tests with absolute alcohol were then performed in a single-blind fashion with the patient fasting. Five hundred ml of 2% absolute alcohol diluted in fruit squash produced widespread urticaria within 15 min. No flushing was seen. A control drink of squash produced no urticaria. This effect was reproducible. Twenty ml of absolute alcohol given via a nasogastric tube produced extensive and severe wealing within 15 min. Normal saline control produced no reaction. Mouth rinsing with absolute alcohol (20 min) and inhalation over a beaker of absolute alcohol (20 min) produced no local or general reaction. Prick tests, scratch tests, and open and closed patch tests with graded strengths of alcohol were all negative, C_1 esterase inhibitor, C_3, C_4 and serum IgE levels were all normal during a positive provocation test.

Treatment with H_1 antihistamines, and a combination of H_1 and H_2 antihistamines did not prevent wealing with alcohol challenge. However, the reaction to alcohol was prevented by indomethacin (25 mg qds 24 h before challenge) and naloxone infusion (0·4 mg/min, −5 to +20 minutes), and sodium cromoglycate. Sodium cromoglycate 100 mg qds has prevented the reaction with alcohol on repeated single blind alcohol challenge. Blood alcohol levels taken over

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a 2-h period following alcohol challenge with and without sodium cromoglycate showed no difference in the pattern of absorption of alcohol.

DISCUSSION

Patients with urticaria may complain that their symptoms are made worse when they drink alcoholic beverages. It is often only specific drinks which cause urticaria, suggesting that a particular component of the drink rather than the alcohol itself produces the reaction. These additives include preservatives, flavouring and colouring agents, and salicylates, which are all well recognized triggers of urticaria (Warin & Champion 1974).

The urticaria in this patient was caused specifically by alcohol. This is a very rare cause of urticaria and has been described in only two patients (Hicks, 1968; Karvonen & Hannuksela, 1976). The blocking of alcohol-induced urticaria by indomethacin, a cyclo-oxygenase inhibitor, and by naloxone, a specific opiate-receptor antagonist, suggests that the urticaria in this patient was mediated by endogenous opiates and prostaglandins. These mechanisms have been suggested as the basis of the chlorpropamide-alcohol flush (Medbak *et al.*, 1981). Alcohol itself may have some opiate-like activities (Schulz *et al.*, 1980) and alcohol coma may be reversed by naloxone (Jeffreys, Flanagan & Volans, 1980). Opiates have been shown to affect the prostaglandin biosynthetic pathway in various tissues including brain (Scoto *et al.*, 1979), and alcohol therefore might enhance or release prostaglandins either directly or by enkephalin release and so initiate wealing due to their vasoactive properties.

We are unable to explain the mechanism of action of sodium cromoglycate in this case. Only 1% of the drug is absorbed and it did not alter the absorption pattern of alcohol during a positive challenge test. However, Soter, Austen & Wasserman (1979) have reported that this drug can ameliorate skin wealing and central nervous system symptoms in systemic mastocytosis without altering histamine levels, suggesting that this drug may have actions other than its known effect on the stabilization of mast cell membranes.

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Brief Communication

Farmyard pox: parapox virus infection in man

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SUMMARY

Inasmuch as orf, milker's nodules and bovine papular stomatitis pox are clinically identical in man and are induced by currently indistinguishable parapox viruses, we propose a new generic term 'farmyard pox' for these diseases. This affords the clinician a diagnosis based on a common set of clinical and electron microscopic findings rather than one based on an uncertain or even misleading history.

A case in point is reported in which the history failed to reveal a specific animal source of the virus, but electron microscopy confirmed the presence of parapox infection.

CASE REPORT

A 35-year-old machinist suddenly developed two boggy, gray, erythema-rimmed bullae on his knuckles. He denied injury or contact with infected sheep, goats, or cows. However, farming was his vocation and while medicating calves with the 'scours', he had introduced medication into their throats with his bare hands. Skin biopsies at 48 and 96 h were non-diagnostic, but electron microscopy disclosed free parapox virions (Fig. 1).

DISCUSSION

The exact animal source of the parapox virus infection could not be identified in the present case. The patient had sheep, but denied direct contact with them or the presence of any scabby mouth in the herd. He did have contact with farm machinery, but had no milk cows or cattle with stomatitis. Possibly the newly-purchased calves had been exposed to pseudo-cowpox while nursing in the mother herd, but he had seen no evidence of a papular stomatitis among them. Therefore, none of the big three farmyard viral entities could be identified by history. Under these circumstances, our proposed diagnosis of 'farmyard pox' seemed appropriate, at once pointing out the parapox viral etiology of his disease and eliminating the need to ascribe it to a specific animal source.

Today, orf, milker's nodules, and the cutaneous form of bovine papular stomatitis are distinguished from each other in man solely by patient history, being essentially identical on clinical, histological and virological examination (Johannessen *et al.*, 1975; Davis & Musil, 1970; Bowman *et al.*, 1981). Orf is diagnosed if the patient has been in contact with sheep or goats,

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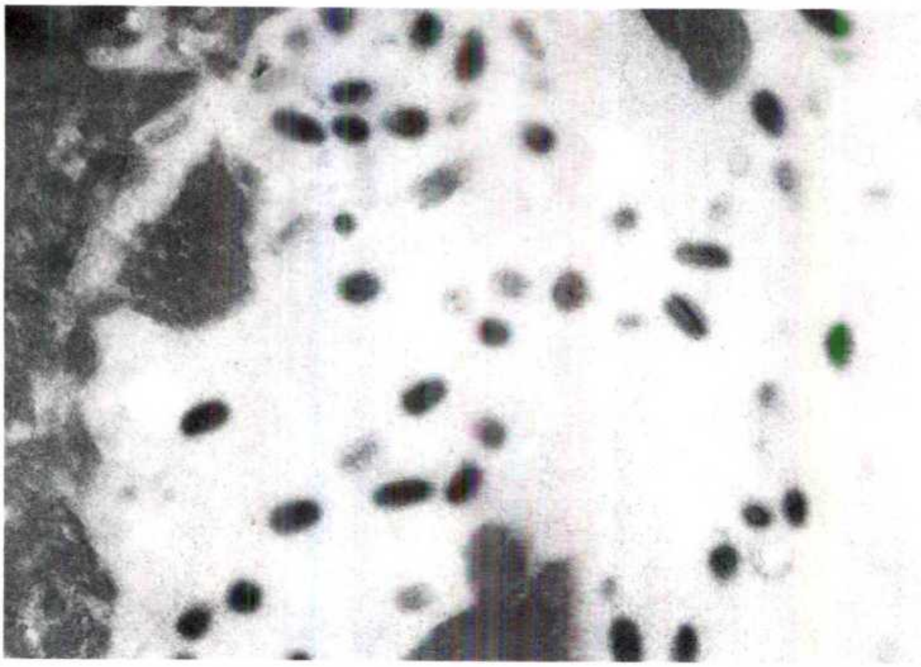

FIGURE 1. Electron micrograph of virions seen in sub-epidermal region of patient's lesion 48 h after onset. These are indistinguishable from those of orf, milker's nodules or bovine papular stomatitis (× 36,000).

whereas milker's nodules are suspected if the patient has been manually milking cows, and bovine papular stomatitis nodules seem likely if the patient has had contact with the oral mucosa of cattle. Often the patient can remember seeing a scabby mouth, pseudo-cowpox of the udder, or papular stomatitis in his farm animals. In the absence of such information, however, it must be assumed that unrecognized active disease was present in the animals or that the patient had contacted inanimate material contaminated with the virus. Even more confusing is the patient who had had both ovine and bovine exposures.

Although in animals the presentation varies, in man the clinical picture of all three is usually the same: discrete pox lesions of the hands, which begin as erythematous macules, progress to purplish granulomatous umbilicated nodules, and spontaneously resolve in 4 to 6 weeks. Occasional lesions have ranged from insignificant pustules to giant epithelioma-like tumours, leading to amputation of affected fingers in at least two individuals (Johannessen *et al.*, 1975; Savage & Black, 1972). Rarely, transitory lymphangitis, lymphadenopathy, fever, or disseminated lesions occur (Wilkinson, 1977).

Electron microscopy of skin biopsies is required to identify free parapox virions within the scale, the cytoplasm of degenerating keratinocytes, and in the oedematous upper dermis. These diagnostic parapox virions are ovoid particles 200–280 nm by 150–250 nm, but they may also be elongated up to 500 nm in length (Nagington, Newton & Horne, 1964; Cheville & Shey, 1967; Nagington, Plowright & Horne, 1962). They must be distinguished from the orthopox virions (vaccinia and variola) which are brick-shaped and somewhat shorter. The parapox virions display unique helical criss-cross banding which is not demonstrable in tissue sections, but only in the free virion negatively stained with phosphotungstate in an acid medium. While subtle tentative distinctions between parapox virus strains have been found using restriction endonuclease analysis (Wittek, *et al.*, 1980), such research typing is neither useful in clinical diagnosis nor available in clinical practice. We believe the term 'farmyard pox', underscores the discrete, vesiculo-pustular lesions characteristic of 'pox' diseases and alludes to its viral aetiology, regardless of presumed animal source.

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Comment

RECENT ADVANCES IN THE IDENTIFICATION OF ENZYME DEFICIENCIES IN THE PORPHYRIAS

Although the porphyrias have long been recognized as disorders of haem biosynthesis, only during the past 12 years has it become apparent that each type results from a partial deficiency of one of the enzymes of haem biosynthesis. Enzyme deficiencies giving rise to porphyrias have now been described for each stage in the conversion of 5-aminolaevulinate (ALA) to haem (Fig. 1 and Table 1) (for reviews see Sassa & Kappas, 1981; Goldberg & Moore, 1980; Schmid, 1982). These defects do not lead to haem deficiency, except possibly in the liver during attacks of acute porphyria, because they are compensated by an increase in the intracellular concentration of the substrate of the defective enzyme which is sufficient to restore the rate of haem synthesis to normal. This increase is brought about by induction of ALA synthetase, the rate-controlling enzyme of the pathway which is under negative feedback control by haem (Fig. 1). It produces the distinctive patterns of accumulation and excretion of porphyrins and their precursors that characterize each of the main types of porphyria (Table 1) and underly many, perhaps all (Rimington *et al.*, 1967; Bonkowsky & Schady, 1982; Varigos, Schultz & Bickers, 1982) of their clinical features.

The first such enzyme defect to be identified was the inherited deficiency of porphobilinogen (PBG) deaminase that causes acute intermittent porphyria (Strand *et al.*, 1970). In this condition PBG deaminase activity is decreased on average to 50% of normal in the liver and in all extra-hepatic tissues, including erythrocytes, that have been investigated. The structural gene for PBG deaminase is situated at a single locus on chromosome 11 (Meisler *et al.*, 1980), and both the autosomal dominant inheritance of acute intermittent porphyria and the extent of the decrease in enzyme activity point to the presence in this condition of a mutant gene, which is not expressed as active enzyme, allelic to the normal gene at this locus. The low, but qualitatively normal, PBG deaminase activity therefore represents expression of the normal allele. At the molecular level, there is genetic heterogeneity with at least two different types of mutation producing the enzyme defect (Anderson *et al.*, 1981).

All other types of porphyria, apart from the very rare autosomal recessive condition, PBG synthase deficiency (Doss *et al.*, 1982), result from defects of enzymes that metabolize porphyrins, or unstable haem precursors that are converted non-enzymatically to porphyrins within tissues, and are thus associated with photosensitization (Fig. 1 and Table 1). Two of these, hereditary coproporphyria and variegate porphyria, are autosomal dominant disorders which share with acute intermittent porphyria the clinical features of acute attacks of porphyria and are also characterized by decreases in enzyme activity to approximately 50% of normal in all tissues, although the enzymes concerned, unlike PBG deaminase, are located in mitochondria and are therefore not present in mature erythrocytes. In hereditary coproporphyria, partial deficiency of coproporphyrinogen oxidase has been established (Hawk *et al.*, 1978), but the localization of the defect in variegate porphyria has been controversial. Reports that ferrochelatase activity is decreased in variegate porphyria have not been confirmed and studies in which both this enzyme and protoporphyrinogen oxidase have been measured in lympho-

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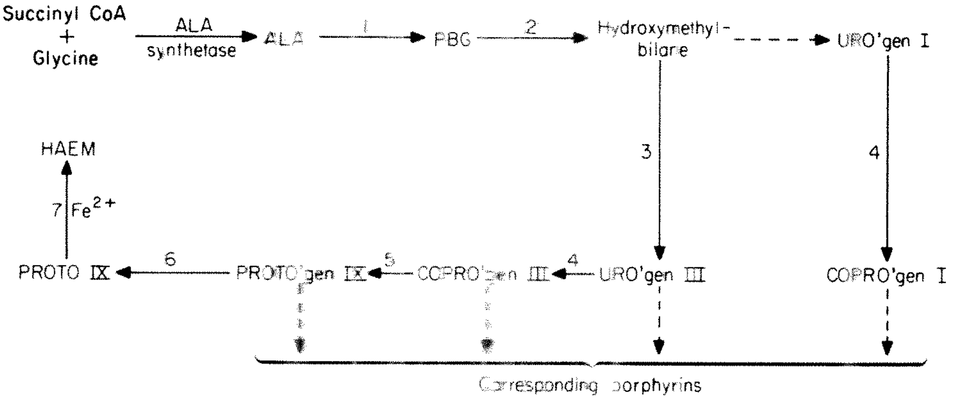

FIGURE 1. Pathway of haem biosynthesis. ALA = 5-aminolaevulinate; PBG = porphobilinogen; URO'gen = uroporphyrinogen; COPRO'gen = coproporphyrinogen; PROTO'gen = protoporphyrinogen. Enzymes are numbered 1–7 as in Table 1. (– – – –) indicates non-enzymatic polymerization of hydroxymethylbilane to uroporphyrinogen I and non-enzymatic oxidation of porphyrinogens to porphyrins.

cytes and fibroblasts strongly suggest that a deficiency of protoporphyrinogen oxidase is the primary inherited abnormality (Brenner & Bloomer, 1980; Deybach *et al.*, 1981). The use of enzyme measurements has also led to the identification of patients who are homozygous for hereditary coproporphyria or a closely related coproporphyrinogen oxidase defect (Grandchamp, Phung & Nordmann, 1977; Nordmann *et al.*, 1983). Two brothers with a form of cutaneous porphyria related to variegate porphyria may similarly be homozygous for a protoporphyrinogen oxidase defect (Stolman *et al.*, 1983). In these homozygous variants, skin lesions may occur in early childhood, whereas in the corresponding single gene disorders these are not seen before puberty.

Primary enzyme defects have also been identified in the purely cutaneous types of porphyria. In the autosomal dominant condition, protoporphyria, ferrochelatase activity is decreased to about 15% of normal in all tissues that have been examined (Bonkowsky *et al.*, 1975; Sassa *et al.*, 1982). At present the unexpectedly low activity for an autosomal dominant enzyme defect has not been explained, and it is unclear whether the measurements reflect *in vivo* activity (Sassa *et al.*, 1982). In the much rarer condition, congenital erythropoietic porphyria (Günther's disease), there is now substantial evidence, including that from enzyme studies on the parents of affected individuals, to indicate that these patients are homozygous for a structural gene mutation that decreases uroporphyrinogen-III-cosynthase activity in all tissues, and leads to overproduction by erythroid cells of type I porphyrin isomers (Nordmann & Deybach, 1982). However, impaired metabolism of the recently identified substrate of this enzyme, hydroxymethylbilane (Battersby *et al.*, 1980) (Fig. 1), has not yet been directly demonstrated. Enzyme studies have failed to reveal any quantitative difference between the usual form of congenital erythropoietic porphyria and the late onset type which presents in adults and is clinically indistinguishable from porphyria cutanea tarda (Deybach *et al.*, 1981).

All the conditions discussed so far show established patterns of inheritance with expression of enzyme defects in all tissues. In the commonest of the porphyrias, porphyria cutanea tarda (PCT), the situation is more complex. The fundamental biochemical abnormality in all patients is undoubtedly decreased uroporphyrinogen decarboxylase activity in the liver (Felsher *et al.*,

TABLE 1. Enzyme deficiencies in the porphyrias

Defective enzyme		Activity in porphyria (% normal)	Condition	Overproduction of:	Inheritance
1. PBG synthase		1–2	PBG synthase deficiency	ALA, coproporphyrin III	Autosomal recessive
2. PBG deaminase		47	Acute intermittent porphyria	PBG, ALA	Autosomal dominant
3. Uroporphyrinogen-III-cosynthase	(i)	16	(i) Congenital erythropoietic porphyria	Uroporphyrin I, coproporphyrin I	Autosomal recessive
	(ii)	15	(ii) Late onset form of (i)		Autosomal recessive
4. Uroporphyrinogen decarboxylase	(i)	41–65	(i) Familial PCT	Uroporphyrin and other acetate-substituted porphyrins‡	Autosomal dominant
	(ii)	40*	(ii) Sporadic PCT		Pathogenesis unknown
	(iii)	not measured	(iii) Toxic PCT		Acquired
	(iv)	5–8	(iv) Hepatoerythropoietic porphyria		(?) Homozygous for familial PCT gene
5. Coproporphyrinogen oxidase	(i)	48–53	(i) Hereditary coproporphyria	Coproporphyrin III†	Autosomal dominant
	(ii)	2	(ii) Homozygous coproporphyria		Homozygous for hereditary coproporphyria gene
6. Protoporphyrinogen oxidase		43–55	Variegate porphyria	Protoporphyrin, coproporphyrin, porphyrin-peptides†	Autosomal dominant
7. Ferrochelatase		13–23	Protoporphyria	Protoporphyrin	Autosomal dominant

PCT = Porphyria cutanea tarda; PBG = Porphobilinogen; ALA = 5-aminolaevulinate.

* Liver only, normal activity in extra-hepatic tissues.

† PBG, ALA during acute attacks of porphyria.

‡ Erythrocyte protoporphyrin concentration increased in hepatoerythropoietic porphyria.

1982) and this appears to be a prerequisite for the development of PCT in response to factors such as alcohol and hepatic siderosis. However, patients with this defect can be divided into two groups by measuring uroporphyrinogen decarboxylase in red cells and other extrahepatic tissues (Verneuil *et al.*, 1978). In one group, extra-hepatic enzyme activity is decreased to about 50% of normal. This defect is inherited as an autosomal dominant characteristic and patients with this type of PCT (familial PCT) may present at any age, including childhood, and often, but by no means always, have a family history of overt PCT (Verneuil, *et al.*, 1978; Elder *et al.*, 1980). In the other group, extra-hepatic enzyme activity is normal (Elder, Lee & Tovey, 1978). Almost all the patients in this group are adults without any evidence of PCT in other members of their families and therefore have been described as sporadic PCT (Verneuil *et al.*, 1978; Elder *et al.*, 1980). In addition to sporadic PCT, this group also includes the form of PCT that may follow exposure to toxic chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Pazderova-Vejlupkova *et al.*, 1981) and hexachlorobenzene which, from experiments in animals, are known to inhibit uroporphyrinogen decarboxylase in the liver but not in red cells. In Europe, South Africa and South America, sporadic PCT is much commoner than familial PCT but, in North America, the incidence of these types appears to be reversed (Kushner, 1982); although more studies are needed from all areas to establish this difference.

PCT is not the only cutaneous porphyria associated with uroporphyrinogen decarboxylase deficiency. In the rare condition, hepatoerythropoietic porphyria, in which skin lesions appear in childhood, the activity of this enzyme is decreased to less than 10% of normal, and family studies suggest that these patients may be homozygous for the gene that causes familial PCT (Elder *et al.*, 1981). A patient, clinically resembling congenital erythropoietic porphyria, with dyserythropoietic anaemia, partial deficiency of uroporphyrinogen decarboxylase, normal uroporphyrinogen-III-cosynthase activity, and overproduction of porphyrins from erythroid tissue, has also been described (Kushner *et al.*, 1982).

Enzyme deficiencies do not always lead to detectable substrate accumulation. This is true both for tissues and for individuals. For example, some enzyme defects may produce compensatory changes (increased substrate concentration and ALA synthetase activity) in the liver but not in erythroid cells: a difference that underlies the division of the porphyrias into hepatic and erythropoietic groups. Also important are the wide differences in response to the same enzyme defect between individuals. Latent porphyria is commoner than manifest disease in all types of porphyria, other than the autosomal recessive conditions, and a proportion of porphyria gene carriers—probably less than 10% in the cutaneous porphyrias—have no detectable biochemical abnormality apart from decreased enzyme activity. Some of the factors that, at least partly, determine these differences have been identified; for example, the effect of barbiturates and other drugs in the acute hepatic porphyrias, and of alcohol and hepatic siderosis in PCT, but in general the relationship between enzyme deficiency and substrate overproduction is poorly understood and is clearly of importance in devising rational methods of treatment.

When should enzyme measurements be requested? Their main use is in family studies and, occasionally, for the investigation of patients in clinical remission, rather than for the diagnosis of overt porphyria which can be effectively achieved by porphyrin and porphyrin precursor measurements. Even so, only PBG deaminase measurements are widely available at present and enzyme tests must of necessity be reserved for suspected gene carriers who show equivocal or normal patterns of porphyrin and porphyrin precursor production. Faced with a patient with acute intermittent porphyria, hereditary coproporphyria or variegate porphyria, the physician is under an obligation to identify relatives at risk from acute porphyria and to warn them about precipitating factors. Enzyme tests allow this to be done before puberty, when haem precursor excretion is normal and acute attacks almost unknown, and for those adults with normal excretion patterns. For protoporphyria and PCT, the position is less clear-cut. Patients and their relatives understandably may wish to know how the defect is distributed in the family, but this knowledge does little to aid management. This situation would be reversed if, for example, means were devised of preventing photosensitivity or liver damage in protoporphyria. In the investigation of PCT, the autosomal dominant type can often only be distinguished from the more usually sporadic type by enzyme measurements. However, it is probably too early to judge the practical value of routinely measuring red cell uroporphyrinogen decarboxylase in all cases of PCT. More needs to be learned about the incidence and natural history of the different types and, for realistic counselling, about the risk of a carrier of the gene for familial PCT developing symptoms. Finally, enzyme measurements may be of great value in investigating atypical forms of porphyria as witnessed by the recent description of homozygous and other variants, and as an adjunct to the prenatal diagnosis of congenital erythropoietic porphyria (Nordmann & Deybach, 1982).

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British Journal of Dermatology (1983) **108**, 735–738.

Correspondence

The mechanism of ultraviolet erythema

SIR, I have been reading the debate on mechanisms of UV-erythema in your journal (see references) with a mixture of amusement and puzzlement. At first sight it seems strange that Shuster started his reply to experts on UV-erythema by explaining the nature of scientific discovery, admittedly, a very fascinating subject. This explanation apparently serves to justify Shuster's contribution to the debate. Shuster argues that discoveries are not made through scholarship but that they are made by the young in their 'blessed state of ignorance' (are the advances in modern science due to a flourishing ignorance among scientists?). This simple attitude towards science apparently explains two things: (a) Shuster selects facts and arguments to support his view (which is all right) and (b) he disregards other interpretations of the same facts and also ignores the facts which support the opposite view (which is not all right).

In his ideas on scientific discovery Shuster confuses an open mind with ignorance. Although the latter can contribute to the former, there is undeniably a distinct difference between the two. Clearly, a scientist should strive for an open mind and fight ignorance. I was surprised to find that Shuster seems to hold the opposite view: he is even flattered when people notice his ignorance.

Shuster's statement that the photographic images produced by UV-erythema rule out a diffusion mechanism is deceptively simple, but nevertheless false. Even after Van der Leun's reply, he appears to have no understanding of the biophysics of epidermal-dermal diffusion. He again tries to counter good *quantitative* measurements and physical reasoning with *qualitative* arguments. Based on physical arguments one would expect the extension of a moderate erythema caused by a hypothetical diffusing substance to be extremely small. Dermatologists are accustomed to observe the epidermis through a microscope, but it should be remembered that it is less than 0·1-mm thick, and that the superficial blood vessels are equally close to the epidermis. Thus, a sharp image of a UV-erythema is readily reconcilable with a diffusion mechanism (note that even the sharpest photographs prove to be unsharp if we enlarge them). Shuster's conception that an observed extension of an erythema is caused by an 'eventual diffusional overflow of vasogoodies' is certainly defendable. But he clearly shows lack of an open mind by stating: 'Van der Leun's quantitative arguments (concerning diffusion) are concerned with what happens when there is diffusional spead and *therefore irrelevant*'. Here Shuster bluntly dictates what is relevant by limiting himself to his pre-assumed mechanism of erythema. The legitimate way to find out whether the diffusion theory is relevant or not, is by comparing it with observations.

Furthermore, I was puzzled by Shuster's remark that it was an 'advance' that Greaves agrees with him on the primary target (viz. the vessels). Beside the fact that it is pleasant for people to agree on a subject, I fail to see a real advance in any scientific sense. My careful conclusion would be that we should not be too sorry that Professor Shuster was *only* passing through photobiology.

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Reply

SIR, Between Van der Leun's hearty reception and de Gruijl's delicate *envoi*, it is possible to see the barriers some would build around the scientific arena. My comments on the nature of scientific discovery were by way of objection to Van der Leun's attempts to distribute entry tickets. Nobody, a singular group to which I have long belonged, needs them. But in truth I find de Gruijl's contribution tedious because it is a reply to something I didn't write. I had already made the very point that a sharp image of UV-erythema is reconcilable with diffusion of a vasoactive agent and went on to discuss the various mechanisms by which such a localization could occur and how the effect of the ubiquitous, diffusable dermal prostaglandins could be localized to vessels in the UV beam, and I ended with the ultimate tease of testability. The argument has not been understood and I suspect that Dr de Gruijl missed it because he suffers from the contemporary disease of science which confuses facts with the reality into which only thought can organize them. Dr de Gruijl must learn the difference betweeen a closed and a focused mind, though no doubt the narrowing of the beam can be confusing.

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SAM SHUSTER

Langerhans cells in lichen planus

SIR, I read with interest the studies by Dr Bhan *et al.* (*British Journal of Dermatology* 1981, **105**, 617) and Drs Ackerman and Ragaz (1981) which report the finding of considerably increased numbers of Langerhans cells in the epidermis of lichen planus using the techniques of monoclonal antibody binding and qualitative light and electron microscopy, respectively. Ackerman & Ragaz concluded that 'the most important new finding was that of Langerhans cells in increased numbers in the epidermis very early in the disease. . .', a conclusion which was reiterated in subsequent correspondence to the *British Journal of Dermatology* (1982, **106**, 613).

Both groups of investigators appear to have overlooked, or perhaps decided not to cite, previous literature which is quite relevant to their studies, and especially to the claim by Ackerman & Ragaz of the 'new finding' of increased numbers of Langerhans cells in the epidermis of early lichen planus. The specific literature is a paper by Paolo Lisi (1973). Using the ATPase activity of Langerhans cells, this investigator demonstrated large quantitative increases in the numbers of Langerhans cells in the epidermis of recent lesions of lichen planus.

In my opinion, the papers by Bhan *et al.* and Ackerman & Ragaz can be criticized for not citing this relevant literature and for thereby not giving appropriate credit to the original observations of Lisi in 1973. This deficiency is a reminder of the responsibility of authors to conduct a thorough search of the literature when claims of new observations are made.

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Reply

SIR, Dr Piepkorn draws attention to the paper in which Paoli Lisi records an increased number of Langerhans cells in 'recent' lesions of lichen planus. Lisi does not define 'recent' nor does he show photomicrographs of any of the lesions. Never did we say that we were the first to observe an increased number of Langerhans cells in lichen planus. On the contrary, we quoted several authors who had previously described an increased number of Langerhans cells in lesions of lichen planus. What we believe we were the first to observe, however, was an increased number of Langerhans cells in the earliest possible visible lesions of lichen planus, i.e. before the appearance of lymphocytes in the epidermis. Dr Piepkorn did not quote us completely. What we actually said was that 'the most important new finding of our study was that of Langerhans cells in increased number in the epidermis very early in the disease *before the appearance of lymphocytes*.' In sum, we commend Paoli Lisi for his observations but maintain that the statements made by us in our article in *The American Journal of Dermatopathology* regarding a new finding are correct.

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ANNA RAGAZ
A.BERNARD ACKERMAN

Reply

SIR, We agree with Dr Piepkorn that authors should conduct a thorough search of the literature when claims of new observations are made. However, we made no claim of 'new observations' with respect to Langerhans cells in our paper, as Ragaz & Ackerman have implied (1982). Although Lisi's paper shows an increase in the number of ATPase-positive cells in the epidermis of lichen planus lesions, all such cells may not be Langerhans cells (White & Krivit, 1965; Zelickson & Mottaz, 1968). Third, it was not the purpose of our paper to review the literature. Our purpose was to use a series of monoclonal antibodies that define T-cell subsets and Langerhans/indeterminate cells in lesions of lichen planus, and in doing so, to document the cell types.

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Skin collagen content

SIR, It was not until the ethanol evaporated from the New Year's atmosphere, that I saw in the December issue the paper of Oikarinen *et al.* (*British Journal of Dermatology* 1982, **107**, 659) on skin collagen in tuberous sclerosis, and read that 'total collagen content, measured as hydroxyproline per wet weight in non-affected skin and in shagreen patches . . . was the same as in control samples . . .' Incredible that it is already 20 years since we first pointed out that expressing skin collagen per wet weight gives a ratio which tells you almost nothing (Bottoms & Shuster, 1963); yet that ratio continues to lull people into the belief that they are measuring collagen *content* of skin. Need it be said yet again, that it takes two to make a ratio, and that an unchanged ratio does not distinguish between an absolute increase, an absolute decrease, or an absolute non-change in the total content of both components of the ratio. That is the very reason we introduced the units of surface area as the reference point for the measurement of total skin *content* of collagen (which can be used equally well for other constituents) (Bottoms & Shuster, 1963; Shuster, Black & MacVitie, 1975).

For what it's worth, I have long suspected that the skin of patients with tuberous sclerosis is thickened and that its collagen content is likely to be increased (due either to the disease or anti-epileptic therapy). The finding of Oikarinen *et al.* (1982) that the proportion of wet mass occupied by collagen is unchanged in no way alters this clinical view, any more than does a normal ratio of collagen to wet weight in the skin of Cushing's Syndrome or acromegaly conflict with the massive decrease in total absolute collagen content found in the former and the monstrous increase shown in the latter when expressed per unit of skin area (Shuster, Raffle & Bottoms, 1967; Black, Bottoms & Shuster, 1972; Black, Shuster & Bottoms, 1973). Roll on 2003!

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An open letter to physicians with physical impairment

SIR: For the past 2 years, we have been involved in compiling a resource directory for physicians with physical impairment. The work is funded by a grant from the St Paul-Ramsey Hospital Medical Education and Research Foundation. The purpose is to list physicians with various physical disabilities who are willing to provide information and referral services to physicians who incur the same disability and need specific information. Existing rehabilitation programmes are simply not equipped to deal with the situation and 'Impaired Physician' services, except for the recently formed 'American Society of Handicapped Physicians' are directed almost exclusively to those physicians with mental illness or drug dependency.

All physicians with physical disability, no matter how small, are encouraged to respond. Information from a doctor with even a minor disability may be of value to another doctor with multiple disabilities. The cornerstone of this project is your participation.

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British Journal of Dermatology (1983) **108,** 739–740.

Book Reviews

Andrews' Diseases of the Skin—Clinical Dermatology.
A.N.Domonkos, H.L.Arnold and R.B.Odam (1982) 7th Edition. Philadelphia: W.B.Saunders. Pp. 1108. Price £45.

It is a failing of dermatologists to believe that they can write a better short text on their subject than their colleagues and as a result there is an abundance of books aimed at the undergraduate or non-expert practitioner with no specialized interest in the subject. But what can be recommended to the interested student, clinical assistant or other gifted amateur? The fact that Andrews' has reached its seventh edition is evidence of its claim to such a position. It is a well-presented book, amply illustrated with black and white photographs which are for the most part helpful and avoid the temptation to exemplify common conditions with over-florid cases. Sensibly, the clinical descriptions come before aetiology and pathology and the references, grouped under subject headings at the end of each chapter, give a good guide to further reading.

There are however criticisms which need to be made. The attempt to deal with individual subjects in depth and succinctly leads to dogmatic statements which one would wish to see substantiated by references which space does not allow to be included. It is possible, for example, to read the section on atopic dermatitis, and be left with the clear impression that it has a proven allergic basis and not realize that there is any association with asthma or rhinitis. Almost every page contains some statement which would raise eyebrows on this side of the Atlantic. Is Winter Itch synonymous with asteatotic eczema? Is Industrial Dermatitis another name for Allergic Contact Dermatitis? Are bedbugs responsible for 90% of cases of papular urticaria? There is also a tendency to retain the use of obscure latin names (Malum perforans pedis, Psoriasis ostracea, Erosio interdigitalis blastomycetica) which have largely been discarded by most practitioners. It is in the discussion of treatment that the book is at its most idiosyncratic. There is great emphasis on the use of corticosteroids by intralesional injection. Few in this country would regard this method as the treatment of choice in lichen planus or 'ideal' for the small lesions of psoriasis, as is advocated, despite the fact that thinning of the skin might be 'severe enough to create a medicolegal problem'.

There is no doubt that the book achieves its aim in covering the whole of dermatology in a concise manner and one would look hard for serious omissions in the topics discussed. At its price it is good value for money but the prospective purchaser should consider seriously whether the additional cost of one of the larger texts would provide a better return for the outlay.

B.R.Allen

Bleeding Disorders. Investigation and Management. G.I.C.Ingram, M.Brozovic and N.G.P.Slater (1982) 2nd edition. Oxford: Blackwell Scientific Publications. Pp. 413. Price £27.50.

The first edition of this book which appeared in 1965 has been accepted by haematologists as being very reputable. The second much altered edition is no doubt all set to emulate it. Written for anyone with a general interest rather than purely for haematologists, there is quite a lot here for dermatologists to refer to. The various coagulation defects may not come our way very often but there are useful chapters on the investigations to do, for example, as screening tests for the various types of purpura. Disseminated intravascular coagulation is discussed and the bleeding disorders associated with general medical diseases, and there is a long chapter on platelet disorders. Dr McGibbon has contributed a useful chapter on dermatological purpura and things which might be mistaken for it, written of course for the general physician and haematologist rather than for the dermatologist. The last 150 pages are devoted to the various tests, from such simple ones as the bleeding time to the more sophisticated. Altogether there are over a thousand references.

R.H.Champion

Colour Atlas of Dermatology. By L.K.BHUTANI (1982) New Delhi: Interprint. Pp. 192. Price RS 135. Available from Meridien Medical Publications, 145a Croydon Rd., Beckenham, Kent. £17.65.

Professor Bhutani's vast experience of clinical dermatology in a country where the range of dermatoses is much greater and the pathology more flamboyant than in Northern Europe, make him the ideal author for a venture of this sort. The book is printed on high quality gloss paper making the colour illustrations sharp and generally of such good colour values that the result is a high degree of realism. Since most of the illustrations have been taken by Professor Bhutani himself, they successfully emphasize what the dermatologist is looking for in a clinical photograph. The text is succinct and informative and includes many brief descriptions of the histopathology with photomicrographs where necessary.

There is some unfortunate confusion in the pagination and legends between plates 21 and 30 which it is hoped can be corrected.

Students and teachers at all levels will find this a very helpful book and it would be a valuable acquisition for a general medical library. The book is heavily subsidised in India and it is to be hoped that the price fixed for the more developed world will keep it within reach of the limited budgets to which we are becoming accustomed.

W.A.D.GRIFFITHS

Thesis received

Serological Diagnosis of Gonorrhoea using Gonococcal Pili as Antigen. A.P.ORANJE (1982) Erasmus University, Rotterdam.

British Journal of Dermatology (1983) **108**, 741–742.

News and Notices

John Milne Memorial Fund

Applications are invited for travelling scholarships funded by the John Milne Memorial Fund. These are for travel to dermatological meetings or to centres which the applicant wishes to visit to increase his/her knowledge and experience either of clinical problems or of research techniques. The scholarships are open to clinicians and scientists for travel during 1984 and the total value will not exceed £450.

Applications, including a full curriculum vitae and reasons for travel, should be sent to Professor R.M.MacKie, University of Glasgow, Department of Dermatology, Anderson College Building, 56 Dumbarton Road, Glasgow G11 6NU, by 1 October, 1983.

Hair Replacement Congress, New York, June 1984

The International Congress for Hair Replacement Surgery will be held at Mount Sinai Medical Center, in New York City, U.S.A., 14–15 June 1984. Registration fee: $450.00. Further information from: D.B.Stough III, M.D., Program Director, The Stough Dermatology & Cutaneous Surgery Clinic, P.A., Doctors Park, Hot Springs, Arkansas 71901, U.S.A.

Fourth International Congress of Dermatological Surgery, Granada, October 1983

The Fourth International Congress of Dermatological Surgery will be held in Granada on 2–5 October, 1983. Further details are available from Dr Vicente Delgado, General Secretary, I.S.D.S. Department of Dermatology, University of Granada, OTECSA-Recogidas, 21–1, Granada, Spain.

Bill Reed International Travelling Fellowships

Three fellowships will be awarded this year as follows:

1. The winner of the Poster Session at the annual meeting of the European Society for Dermatological Research will be awarded $800.00 to help support a trip to attend the annual meeting of the American Academy of Dermatology.
2. The winner of the Stelwagon Prize for the best paper presented at the Resident's Forum at the annual American Academy of Dermatology meeting will receive an $800.00 award to support a trip to Europe, where the awardee will present a paper at the annual meeting of the British Association of Dermatologists in England. Further travel to European centres can also be arranged.
3. A third award of $800.00 will be given to an academically oriented dermatologist in the private practice of Clinical Dermatology, who has been out of training no more than 5 years. It is meant to support travel for educational purposes and to broaden contacts with dermatological investigators. Anyone interested in applying for this award should send their curriculum vitae and a covering letter indicating how they plan to spend the award funds, to the Chairman of the Committee on International Affairs, A.A.D. Academy Office, 1567 Maple Avenue, Evanston, Illinois 60201, U.S.A. The deadline for submission is 1 September 1983. Supporting letters from abroad relevant to the planned travel are desirable. The award will be announced in December and will be based on the originality and productivity of the applicant as well as the merit of the travel plans for continued clinical investigation.

Correction

E.J.Kraai *et al.*, Cutaneous malignant epidermotropic lymphoma, possibly of monocytic origin. *British Journal of Dermatology* 1983, **108,** 120. Owing to a printing error, two lines were changed in this paper:

Enzyme histochemistry. The tumour cells showed a paranuclear area with granular acid phosphatase activity. Other stains with non-specific esterase, peroxidase and aminopeptidase were negative.

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gram(s)	g	minute(s)	min	molar	mol/l
kilogram(s)	kg	centimetre(s)	cm	millilitre(s)	ml
milligram(s) (10^{-3}g)	mg	second(s)	s	gravitational acceleration	***g***
microgram(s) (10^{-6}g)	μg	cubic millimetre(s)	mm^3	micrometre(s)	μm
nanogram(s) (10^{-9}g)	ng	millimetre(s)	mm	per cent	%
picogram(s) (10^{-12}g)	pg	millicurie(s)	mCi	isotopic mass number placed as	^{131}I
hours(s)	h	milliequivalent	mmol		

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